EVALUATION OF IMMUNITY AND PROTECTION INDUCED IN PULLETS BY THE V4 ORAL VACCINE AGAINST A PNEUMOTROPIC VELOGENIC NEWCASTLE DISEASE VIRUS (NDV) STRAIN

BY

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DEDICATION

To my father Issaca Johane Magalo Chadreque who passed away on 18th November 1999 for his encoragement and educational support.

DECLARATION

I declare that this dissertation which I hereby submit for the degree of Mmed vet (Altil) at the University of Pretoria, is my own work and has not previously submitted by me for a degree at another Univesity.

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Simone Issaca Magalo BSc (Maputo) Date

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SUMMARY

Evaluation of immunity and protection induced in pullets by the V4 oral vaccine against a pneumotropic velogenic Newcastle disease virus (NDV) strain

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Key words: Newcastle disease (ND), Newcastle disease virus (NDV), V4 and ND V4-HR vaccines, B1172 NDV, antibody response, protection, replacement pullets, Haemagglutination Inhibition test and Enzyme-linked immunobsorbent Assay.

Newcastle disease (ND), caused by Newcastle disease virus, is an acute, contagious and pathogenic infection of pet, free living and domestic birds. ND is an epidemic disease and it is responsible for high economic losses due to up to 100 % mortality. The control of ND in the intensive commercial poultry farms is largely dependent on prophylactic immunisation using conventional vaccines.

The ND V4 vaccine and its derivative ND V4-HR vaccine were selected originally for use in village chickens, due to their immunogenicity, thermostability,

transmissibility and ease of administration. The efficacy of V4 and V4HR vaccines have been established in many Asian and African countries in their ability to challenge a wide range of recognised and local velogenic NDV. Therefore, ND V4 was tested for efficacy against B1172 challenge NDV isolated in south Africa in 1993.

Twenty-eight one day-old replacement pullets were vaccinated by eye-drop route at 21 and 49 days old. Chickens vaccinated by eye-drop route were left to mingle with the unvaccinated in-contact chickens. At 63 days all chickens including the unvaccinated control group were individually challenged with B1172 NDV. Serological monitoring of NDV antibody response was done using HI and ELISA tests.

The ND V4 vaccine induced full protection against B1172 NDV in chickens vaccinated by eye-drop vaccination and in 55 % of chickens vaccinated by the in-contact method. No association was seen between NDV antibody titer at pre-challenge and the ability to withstand B1172 challenge NDV.

A fair to good agreement was seen between the HI and ELISA test in monitoring NDV antibody response during the experiment. Although, the ELISA showed a higher sensitivity and specificity than the HI test, further studies are required using this method of comparison.

OPSOMMING

Sleutelwoorde: Newcastle-siekte (ND), Newcastle-siektevirus (NDV), V4 en ND V4-HR entstowwe, teenliggaamrespons, beskerming, vervangingshenne, Hemagglutinasie-Inhibisietoets en ELISA.

Newcastle-siekte(ND) wat deur die Newcastle-siektevirus (NDV) veroorsaak word, is ’n akute, besmetlike en patogene infeksie siekte van troetel-, vrylewende en huishoudelike voëls. ND is ’n epidemiese siekte en is verantwoordelik vir geweldige ekonomiese verliese met ’n mortaliteit van tot soveel as 100%.

Die ND V4 entstof en sy derivaat, ND V4-HR, is oorspronklik weens hul immunogenisiteit, termostabilitiet, oordaagbaarheid en gemak van toediening gekies vir die gebruik in hoenders in ontwikkelende areas. Die doeltreffendheid van V4 V4-HR entstowwe is in verskeie lande in Asië en Afrika gebruik. Hul vermoë om ’n wye reeks van erkende en plaaslike velogeniese NDV te dek is bevestig. Daaron is ND V4 vir doeltreffendheid teen B1172 NDV, wat in 1993 in Suid Afrika geïsoleer is, getoets.

28 dagoue vervangingshennetjie is per oogdruppelroete op dae 21 en 49 geënt. Hoenders wat per oogdruppelroete geënt is, is toegelaat om met oningeënte hoenders te meng . Na 63 dae is alle hoenders, insluitende die oningeënte kontrolegroep, individueel met B1172 NDV gedaag. Die NDV teenliggaamresponse is serologies gemonitor met behulp van HI en ELISA.

Die ND V4 entstof het volle beskerming teen B1172 NDV in hoenders wat per oogdruppel ingeënt is en 55% van die hoenders wat met kontak ingeënt is gebied. Daar is geen verband waargeneen tussen die NDV teenliggaamtiter voor daging en die vermoë om ’n daging met B1172 NDV te weerstaan nie.

Daar was ’n redelike tot goeie ooreenkoms tussen die HI en ELISA toetse in die monitering van die NDV-teenliggaamresponse gedurende die eksperiment. Alhoewe die ELISA egter ’n groter sensitiwiteit en spesifisiteit as die HI toets getoon het, word verdere studies met hierdie metode van vergelyking benodig.

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ABBREVIATIONS

AE	Aerosol challenge method
Aggl	Agglutination test
AGP	Agar-Gel-Precipitation test
B1172/93	South African field NDV isolate
B-Cells	Bursal dependent lymphocytes
BLP	Buffer Lactose Peptone
CAV	Chicken anaemia virus
CE/PM	Clinical examination/post mortem
CMI	Cell-mediated immunity
CO_2	Carbon dioxide
CVL	Central Veterinary Laboratory$^{-Weybridge}$
df	Degree of freedom
ED	Eye-drop challenge method
EID	Embryo infectious doses
ELISA	Enzyme-immunosorbent assay
F	"F" strains of NDV
F	Fisher exact test (Two tailed P- value)
F0	Precursor fusion glycoprotein molecule of NDV
F1 and F2	Pprogenies fusion glycoprotein molecules of NDV
FAO	Food and Agricultural Organisation
Group A	Eye vaccinated group
Group B	In-contact vaccinated group
Group C	Unvaccinated control group
GTZ	German's Technical Co-operation Agency
HA	Haemagglutination activity
HA^-	Negative haemagglution erythrocytes
HA^+	Positive haemagglution erytrocytes
HI	Haemagglutination inhibition (HI)
HR	Heat resistant vaccine
IBD	Infectious Bursal disease
IC	in-contact challenge method
ICPI	Intracerebral pathogenicity index
IgA	Immunoglobulin type A
IgG	Immunoglobulin type G
IgM	Immunoglobulin type M
IM	Intramuscular challenge method
IN	Intranasal challenge method
INIVE	National Veterinary Research Institute of Mozambique
IPVI	Intravenous pathogenicity index test
K agreement	Kappa agreement
Kg	Kilogram
Log_{10}	Logarithms of base 10
Log_2	Logarithms of base 2

m	Meter
m^2	Square meter
MAbs	Monoclonal antibodies
MDT	Mean death time
mL	millilitre
ND	Newcastle disease
ER	Excluding results
NDV	Newcastle disease-virus
NH3	Ammonia
NI	Naturally infected challenge method
NVNDV	Neurotropic velogenic NDV
O	Oral challenge method
P >	Probability to be more
P<	Probability to be less
PBS	Phosphate-buffered isotonic saline
PCR	Polymerase chain reaction
ppm	Parts per million
PRL	Poultry Reference Laboratory
S.A.	South Africa
SABS	South Africa Bureau of Standards
SAPA	South Africa Poultry Association
SD	Standard deviation
Sig	Statistical significance
Sp	Spray challenge method
SPF	Specific pathogen free
Std	Standard
T-Cells	Thymus dependent lymphocites
TNTC	To numerous to count
t-test	Student t-test
UNDP	Union Nations for Development Programmes
UP	University of Pretoria
V4	Australian V4 vaccine
VN	Virus neutralisation test
VVNDV	Viscerotropic velogenic NDV
µL	microliter
X^2	Chi-square
°C	Celsius degrees
+	Positive
-	Negative

CHAPTER 1: INTRODUCTION

Newcastle disease (ND) is an acute, contagious infection of pet, free living and domestic birds. The causative agent is Newcastle disease-virus (NDV), which belongs to the genus *Rubulavirus* and falls into the *Paramyxovirinae* subfamily of the family *Paramyxoviridae* (Alexander 1997). NDV is a worldwide distributed virus (either as naturally circulating virus or as a vaccine virus), and established in at a least 241 species of birds representing 27 of the 50 orders of the class (Alexander 1995b). ND is widely variable in type and severity of the disease it produces. ND is complicated because different isolates and strains of the virus may induce variations in the severity of the disease, even in a given host such as the chicken (Alexander 1991). A variety of NDV isolates and strains have been recorded around the world (Alexander 1991; Ballagi-Pordány, Lagerkvist, Wehmann, Hercezeg, Baranyi, Landegren, Belák & Lomniczi 1995; Ballagi-Pordány, Wehmann, Hercezeg, Belák & Lomniczi 1996; Alexander 1997.). The term “strain” is used here to mean a stable and well-characterised virus (Coetzee 1980; Alexander 1991).

ND is an epidemic disease in intensive poultry and is responsible for high economic losses with up to 100 % mortality (Alexander 1991; Awan, Otte & James 1994). Moreover, ND is recognised as an enzootic disease in most countries of Africa and Asia and some countries of Europe (Awan *et al.* 1994; Alexander 1995a,b, and Ballagi-Pordány *et al.* 1995,1996).

In South Africa (S.A.), ND became enzootic in 1971 and the control of the disease was largely dependent on prophylactic immunisation (Coetzee 1980; Shane 1984). A variety of velogenic NDV isolates have been made by the Poultry Reference Laboratory at the Section of Poultry Health in the Department of Production Animal Studies, Faculty of Veterinary Science of the University of Pretoria (UP) in S.A.

A serious outbreak of ND, characterized by severe haemorrhage of the trachea occurred on a South African poultry farm in 1993 (Coetzee personal-communication 1993) and resulted in a mortality rate of 90 %. A pneumotropic velogenic NDV strain was isolated from this outbreak. This isolate of NDV spread rapidly and a number of other isolations of this virus were made throughout the country. Tests were carried out at the ND-Reference Laboratory (Central Veterinary Laboratory, Weybridge, United Kingdom) to type and characterise the virus isolated from this field outbreak. The isolate showed a positive haemagglutination inhibition (HI) test with Paramyxovirus-1 antiserum and with monoclonal antibodies (MAbs) specific for most classical strains of NDV. A negative HI test result was obtained with MAbs specific for the pigeon NDV, La Sota and "F" strains of NDV. The intravenous pathogenicity index test (IVPI) was 2.17. The NDV isolated from the 1993 outbreak was designated B1172/93 strain and was freeze-dried and stored at the Poultry Reference Laboratory.

The control of ND relies on the use of safe and effective vaccines. Live vaccines prepared with lentogenic or mesogenic strains of NDV are now more commonly used in broilers than vaccines prepared from chemically inactivated strains of

NDV, mixed with adjuvant (Biggs, Box, Brown, McConnell, McFerren & Soulsby 1988; Alexander 1991). This is because live freeze-dried vaccines can be produced on a large scale at a relatively low cost, they are easy to administer on a large scale, and rapidly stimulate humoral, cell-mediated and mucosal surface immunity.

The Webster's[1] ND V4 and ND V4 heat resistant (HR) vaccines are live vaccines based on the Australian V4 strain of NDV (Simons 1967). These vaccines have been widely used in Africa and Asia due to their adequate immunogenicity, thermostability, avirulence and rapid transmissibility (Heath, Lindsey, McManus & Claxton 1992; Spradbrow 1993/4).

In rural areas, especially in village chickens, vaccination efficiency is impaired by heat sensitivity of other vaccines, lack of viable cold chain and cold storage facilities and inability to protect the small, multi-aged flocks scattered over extremely large areas. The ND V4 vaccine has been developed specifically for use in tropical climates in order to reduce the need for the cold chain for vaccine transport. This vaccine is easier to administer by all conventional routes and by mixing in feed (Heath *et al.* 1992; Spradbrow 1993/4). Many tropical countries have officially approved the use of the ND V4-HR vaccine especially in rural areas (Spradbrow 1993/4).

[1] Arthur Webster Pty Ltd, 23 Victoria Avenue, Castle Hill, NSW 2154, Australia

The efficacy of the ND V4 and V4HR vaccines was established in Australia (French, George & Percy 1969; Turner & Kovesdy 1974; Turner, Hanson & Spalatin 1976; Turner, Spalatin & Hanson 1977; Westbury, Parson & Allan 1984; Samuel & Spadbrow 1991), in Africa (Sagild & Spalatin 1982; Sagild & Haresnape 1987; Jagne, Aini, Schat, Fennell & Touray 1991; Alders, Inone & Katongo 1994; Bell, Fotzo & Agebede 1995) and in Asia (Spadbrow, Ibrahim, Mustaffa-Babjee & Kim 1978; Spadbrow, Ibrahim, Chulan, Milliken, Schapcott & Kingston 1980; Ibrahim, Chulan & Babjee 1987; Jayawardane, De Alwis & Bandara 1990; Ideris, Ibrahim & Spradbrow 1990; Bell, Nicholls, Norman, Ideris & Cross 1991a,b). In these countries, the ND V4 and V4-HR vaccines demonstrated adequate immunogenicity and induced protection against a wide range of velogenic strains of NDV. The protection levels reported in these countries ranged between 30 and 100% depending on the virulence of the challenge strains used and the route of administration.

The presence of NDV carrier chickens and a constant introduction of susceptible birds and other poultry species, including wild birds (Awan et al. 1994) influence current velogenic outbreaks of ND in African countries. These factors have contributed to the epidemic appearance of ND in poultry production in rural areas of the African region as well as in commercial operations. The ND V4 and V4HR vaccines have overcome many of the technical and social problems that currently limit the immunisation of village chickens.

Infections with NDV (either naturally or NDV vaccines) may induce cell-mediated immunity, humoral immunity, local immunity and passive immunity

(Alexander 1991,1997). Humoral immunity can be detected and measured by several serological tests (Tizzard 1982; Alexander 1991). Serological testing for antibody to NDV has primarily utilised either the hemagglutination inhibition (HI) test or virus neutralisation test (VN). The HI has been used as the standard test. Recently, enzyme-immunosorbent assay (ELISA) has replaced the HI test (Adair, McNulty**,** Tood, Connor & Burns 1989; Brown, Resurreccion & Dickson 1990; Alexander 1991).

Several serological studies have been conducted to evaluate the relationship between ELISA and HI tests for NDV (Miers, Bankowski & Zee 1983; Snyder, Marquart, Mallinson & Russek 1983; Marquardt, Snyder, Savage, Kadavil & Yancey 1985; Thayer, Villegas & Fletecher 1987; Thayer, Nersessian, Rivetz & Fletcher 1987; Adair *et al.* 1989; Brown *et al*. 1990; Czifra, Nilsson, Alexander, Manvell, Kecskemétt & Engström 1996; Czifra, Mészáros, Horvath, Moving & Engström 1998).

The objectives of this research project were to provide further information of immunity and protection induced by the ND V4 vaccine against the B1172/93 strain of NDV isolated in South Africa in 1993 and to compare the HI-NDV and ELISA-NDV tests with respect to the detection of NDV antibodies induced during the experiment

CHAPTER 2: LITERATURE REVIEW

Several studies have reported the efficacy of ND V4 and 4-HR vaccines (Heath *et al.*1992, Spradbrow 1993/4; Jayawardane & Spradbrow 1995a,b; Bell *et al*. 1995). Chickens vaccinated with ND V4 and V4HR vaccines and possessing low levels or undetectable levels of serum HI antibodies against NDV resisted challenge with virulent strains of NDV. The mucosal immune system, in addition to humoral immune responses was thought to be involved in the immunity and protection induced by ND V4 vaccine (Turner *et al.* 1976; Spradbrow *et al.* 1978; Ibrahim *et al.* 1987; Spradbrow & Samuel 1991, 1992; Spradbrow 1993/4; Jayawardane & Spradbrow 1995b). Therefore a discussion of the mechanism underlying the avian immune response is important to understanding how this vaccine induces protection against velogenic pathotypes of NDV.

2.1. Pathogenicity of NDV

The pathogenicity of NDV isolates and strains varies markedly with the host (Alexander 1991). Chickens and turkeys of all breeds are susceptible to NDV. Ducks and geese have shown some resistance to NDV while wild birds, caged "pet birds", and racing birds are susceptible to NDV (Alexander 1995b). Other factors (species, age, immune status of the host, co-infection with other organisms, environmental stress, social stress, route of exposure and the virus dose) play a role in the pathogenicity of NDV (Parede & Young 1990; Alexander 1991).

Several methods have been used to group isolates and strains of NDV. Hansen and Beard (1967 cited by Alexander 1991), suggested five pathotypes of NDV based on disease produced under laboratory conditions:

1) Viscerotropic velogenic NDV (VVNDV), the group of NDV causing acute, lethal infections of chickens of all ages, with evidence of haemorrhagic lesions of the digestive tract,

2) Neurotropic velogenic NDV (NVNDV), those producing acute, often lethal infections in chickens of all ages following respiratory and neurological signs,

3) Mesogenic NDV, less pathogenic than NVNDV and causing respiratory and sometimes nervous signs with low mortality,

4) Lentogenic NDV, which is characterized by mild or inapparent respiratory infection,

5) Apathogenic or asymptomatic enteric NDV, those causing an inapparent gut infection.

At present, there are three widely used laboratory methods for testing the pathogenicity of the virus of ND (Alexander 1988,1991, 1997):

1) The mean death time (MDT) in 9-10 day-old chicken embryo eggs. The MDT pathogenicity test groups NDV into velogenic, mesogenic and lentogenic

types based on the chicken embryo mortalities occurring before 60 hours (h), between 60 and 90 h and after 90 h respectively, after allantoic inoculation.

2) The intracerebral pathogenicity index (ICPI) in day-old chickens. This test involves inoculation of NDV infectious allantoic fluid into the brain of 10 one-day-old SPF chicks and each bird is observed at 24-hour intervals. Each bird is scored as “0” if normal, “1” if sick and "2" if dead. The ICPI is the mean score per chick observation over 8 days. Less virulent ND viruses give an ICPI value up to 0.4. Mesogenic ND viruses show an ICPI around 1.4 while the most virulent ND viruses have an ICPI value of up to 1.7 (Alexander 1995a)

3) The intravenous pathogenicity index (IVPI). This test comprises the intravenous inoculation of NDV derived from fresh infectious allantoic fluid into 10 six-week-old SPF chickens and each bird is observed over 24 hours. Each bird is scored as “0” if normal, “1” if sick and “2” if paralysed and “3” if dead. The IVPI is the mean score per chick observation over 10 days. The most virulent ND viruses show values close to 3.0 whereas those of low virulence and most of the intermediate virulence have values of 0.0.

Strains and isolates of NDV are also grouped on the basis of MAb technology (Alexander, Manvell, Parsons, Colins, Brockman, Russell & Lister, 1987 cited by Alexander 1991; Russell & Alexander, 1983 cited by Manvell, personal communication 1993). MAbs detect variation in antigenicity such as single amino acid changes at the epitope to which the antibody is directed (Alexander 1991).

Thus, the MAb technology places strains and subpopulations of NDV into groups on the basis of their ability to react with different MAbs

More recently, Ballági-Pordany *et al.* (1995, 1996) described the use of polymerase chain reaction (PCR) as the simplest method for grouping NDV strains into different lineages.

The mechanism underlying the pathogenicity of the different strains of NDV has been explained on a molecular basis (Alexander, 1991). The precursor fusion glycoprotein F0 molecule must be cleaved into F1 and F2 for the progeny virus to become infective to the host cell. This linkage has demonstrated to be susceptible to cleavage by a wide range of host proteases. Thus, velogenic NDV replicates in a wide range of organs and tissues of animal origin. However, the presence of the single arginine without a complementary basic amino acid at the cleavage site of the F0 molecule in the lentogenic NDV means that limited cleavage can only be made by host proteases that recognize the single arginine amino acid. Therefore, F0 molecules of avirulent NDV are restricted to the site of the host proteases for their replication. They only replicate in limited host proteases (such as trypsine-like enzymes) associated with the mucosal surfaces of the respiratory and intestinal tracts (Alexander 1991). Therefore the mucosal surfaces of the respiratory and intestinal tracts constitute the natural routes whereby NDV gains access to the host.

2.2. Avian Immune Response

Several researchers have reviewed the mechanism involved in the avian immunity (Toivane & Toivane 1987; Vainio & Toivanen 1987; Alexander 1991; Sharna 1991). Sharna (1991) stated that, the avian immune system is governed by the same principles as the mammalian immune system in that cellular cooperation exists between macrophages and lymphocytes and the interplay between T-cells and B-cells is of critical importance. The T-lymphocytes are components of cellular immunity while the B-lymphocytes constitute humoral immunity (Toivanen & Toivanen 1987).

Like other viruses, NDV may induce cell-mediated immunity, humoral immunity, local immunity and passive immunity. Immunosuppressive diseases can influence the immune response induced by NDV infection, either naturally or by vaccines (Alexander 1991; Sharna 1991).

2.2.1. Passive Immunity

By definition, passive immunity is the transfer of maternal antibodies from the mother to her progeny and is important for early protection of the offspring (Alexander 1991). Passive immunity may interfere with the immune response to vaccination when live vaccines are used. It involves immunoglobulin type G (IgG) but not immunoglobulins type A (IgA) or immunoglobulin type M (IgM) (Darbyshire 1987; Alexander 1991).

Hens, with antibodies to NDV pass these to their progeny via the egg yolk and they take up to 4.5 days after hatching to be demonstrated by HI testing (Alexander 1991).

2.2.2. Cell Mediated Immunity

T-cell-mediated immunity (CMI) by definition, is the antibody independent immune system that is under control of the thymus (Schultz 1982; Toivane & Toivane 1987)). It provides an initial immune response to infection, and can be detected as early as 2-3 days after vaccination with live vaccines (Ghuman & Bankowski 1975, cited by Alexander 1991; Timms & Alexander 1977; Agraval & Reynolds 1991; Jayawardane & Spradbrow 1995a).

In the case of NDV infection, infected cells can be lysed by sensitised lymphocytes when they recognise the viral antigens on surface receptors of cells where the replication of NDV has occurred. Soluble NDV antigens may also induce the sensitised lymphocytes to release lymphokines that may amplify both the local immune response and the humoral immune response (Timms & Alexander 1977; Slauson & Cooper 1984; Schat 1991, Russell 1993).

2.2.3. Local Immunity

Local immunity is an integral part of total immunity, in addition to CMI and other humoral factors, for early protection. It involves not only IgA (Timms & Alexander 1977; Baba, Kawata, Masumoto & Kajikawa 1990; Alexander 1991; Russell

1993, Russell & Kock 1993) but also locally synthesized IgG and IgM in the Harderian gland (Russell, 1993; Russell & Kock 1993). IgA is intimately associated with the mucosal surface secretions. It is associated with the mucosa of the upper respiratory tract (Aitaken & Parry 1976), intestinal tract (Zigterman, Van de Ven, Van Geffen, Loeffen, Panhuijzen, Rijke & Vermeulen 1993) and Harderian gland (Russell & Kock 1993).

The mechanism of locally induced immunity is unknown (Alexander 1991). However, there is strong evidence that it is better correlated with resistance to infection than is the humoral immune response.

In NDV infection, local immunity is induced naturally or with live NDV vaccines incorporating lentogenic or apathogenic strains (Russell 1993; Russell *&* Kock 1993; Jayawardane & Spradbrow 1995b).

2.2.4. Humoral Immunity

Humoral immunity, by definition, is the resistance mediated by cellular immune system that (B-lymphocytes) is under control of the bursa of Fabricius. Activated B-lymphocytes become plasma cells and these secrete blood-derived immuglobulins IgM and IgG (Tizzard 1982; Spradbrow & Samuel 1991). The IgM and IgG are developed sequentially in the process of the humoral immune response (Darbyshire 1987).

Methods for the measurement and detection of humoral immune response (Tizzard 1982; Beard 1989; Alexander 1991) include virus neutralization tests (VN), plaque neutralization, single radial immunodiffusion, single radial hemolysis, agar gel precipitation and enzyme-linked immunosorbent assays (ELISA). The VN response appears to parallel the HI response, which has been used as a standard test for measuring humoral immune response induced by strains of NDV, (Snyder *et al*. 1983; Miers *et al*. 1983; Adair *et al*. 1989; Alexander 1991). However the development of commercial ELISA-NDV kits has made this test more popular than other conventional tests for monitoring many avian diseases simultaneously, including NDV (Alexander 1991).

2.2.5. Immunosuppression

Tizzard (1982), Alexander (1991) and Sharna (1991) have reviewed immunosuppression. Several agents such as infectious bursal disease (IBD) virus, chicken anaemia virus (CAV) and lymphoid leucosis virus induce immunodepression. The mechanism(s) underlying avian immunosuppression are not well understood. Immunosuppression involves the breakdown of the regulatory control mechanism of the immune system (Biggs et al. 1988, Alexander 1991, and Sharna 1991). Immunodeficiency may explain the more severe disease outbreaks and economic losses provoked by some NDV strains and a failure to respond well to vaccination with NDV vaccines (Alexander 1991). Sharna (1991) emphasized that flocks exposed to immunosuppressive agents performed poorly and succumbed to opportunistic infections.

2.3. Protection induced by V4 and ND V4-HR vaccines against several velogenic strains of NDV

Webster's ND V4 vaccine (Webster, Taylor & Barnes 1970) and ND V4HR vaccine (Aini, Ibrahim, Spradbrow & Seng 1987, cited by Bell, Fotzo, Amara & Agbed 1995) are live ND vaccines produced from the apathogenic enteric V4 strain of NDV identified and typed by Simons (1967) in Australia. The avirulence of the ND V4 strain combined with its transmissibility and high immunogenicity prompted the use of the virus strain as a vaccine (Webster, Taylor & Burnes 1970).

The ND V4-HR virus strain was selected from the original ND V4 virus strain and seemed to be a similar immunogen to V4 strain of NDV (Ideris, *et al.* 1990; Jagne *et al* 1991; Spradbrow 1993/4). The ND V4-HR virus strain remains viable up to 56 °C and is stable for long periods on food pellets stored at 25 °C and 4 °C (Ideris *et al.* 1990). Therefore, it is suitable for use as a vaccine destined for distribution to rural areas of tropical countries where the cold chain is difficult to maintain.

The ND V4 and V4-HR vaccines have been shown to protect against a range of velogenic NDV strains. The level of protection induced by the Webster's V4 and ND V4-HR vaccines against a variety of velogenic challenge strains is summarized in Table 2.1.

Table 2.1. Protection conferred by Webster's V4 and V4HR vaccines

Challenge strain of NDV	challenge Method	Protection level(%)	Tested ND vaccine	Reference
Albiston-Gorrie	IM[1]	95 & 100	V4	French *et al.* 1969
Herts 33	IM,	100	V4	Webster 1970
Roakin	O[2], IN[3], IC[4], IM	99	V4	Turner *et al.* 1976, 1977
Texas GB	IM, O	99	V4	Turner *et al.* 1976, 1977
Herts 33	IM, O	100	V4	Turner *et al.,* 1976, 1977
Fontana 1083	IN	100	V4	Spalatin *et al. 1976*
Ipoh AF2240-226	IM	91	V4	Spradbrow *et al.* 1978
Ipoh AF2240-226	AE[5], Sp[6]	96	V4	Ibrahim *et al.* 1987
Ipoh AF2240-226	NI[7]	98-100	V4	Spradbrow *et al.* 1980
Malawian 129/77	ED[8]	100	V4	Sagild & Spalatin 1982
Herts 33/56	IM	100	V4	Westbury 1984
Malawian Strain	ED	100	V4	Sagild *et al.* 1987
Ipoh AF-2240-226	IN, IC	80-100	V4-HR	Ideris *et al.* 1990
SL88/1	IN, IC	66 & 100	V4-HR	Jayawardane *et al*. 1990
Ipoh AF 2240-226	IM, IN, IC	80 & 97	V4	Bell *et al.* 1991
Zambian strain	IN, IC	100	V4-HR	Alders *et al*. 1994
Italian	ED, IC	100	V4-HR	Bell *et al. 1995*

1: intramuscular. 2: oral. 3: intranasal. 4: in-contact. 5: aerosol. 6: spray. 7: naturally infected. 8: eye-drop.

Several workers reported that transmission of V4 and V4-HR ND viruses may occur between vaccinated chickens and in-contact unvaccinated chickens (Bell *et al.* 1991a,b; Samuel & Spradbrow 1991; Spradbrow & Samuel 1991; Heath *et al*. 1992; Spradbrow 1993/4).

Spalatin *et al*. (1976), Sagilg & Harnesnap (1987), Samuel & Spradbrow (1991), Spradbrow & Samuel (1991) Spradbrow (1992) and Alders *et al.* (1994) observed that a wide number of factors including the immune status of the host, the route of exposure, age, temperature, humidity, ventilation and configuration of physical facilities (cages, flooring, etc.) affected the rate of transmission of the ND V4 strain.

The Webster V4 and V4-HR ND vaccines can be administered by all conventional routes of vaccination as well as oral vaccination mixed in food (Samuel & Spradbrow 1991; Heath *et al*. 1992; Spradbrou 1993/93).

The lack of correlation between the antibody titer in chickens vaccinated with V4 and V4HR vaccines and the level of protection to challenge against velogenic NDV has been observed by many authors. Turner *et al.* (1976) demonstrated that chickens possessing no detectable antibody titres or a HI titer $Log_2 \leq 2$ or $Log_2 > 2$ after vaccination with ND V4 vaccine survived challenge with a velogenic NDV. Similar observations were also reported by others authors including Spalatin , Turner *et al*. *(1976),* Spradbrow *et al.* (1978), Ibrahim *et al.* (1980), Spradbrow & Samuel (1991*)* and Jayawardane & Spradbrow (1995a,b).

2.4. Correlation between HI test and ELISA for the detection of antibody to ND

The HI-NDV test has been used as the standard method for detecting antibodies against ND (Alexander 1991). Because of its simplicity, it does not require highly trained personnel or expensive equipment. However, the HI test suffers from several disadvantages. Difference of time in the incubation of antigen/antiserum (Alexander 1997; Maas, Kemper, Koch & Visser 1998), and the positive-negative cut-offs difference (Czifra *et al*. 1998) among laboratories attempt for the lack of the reproducibility of HI test in quantifying protection against several NDV isolates (Miers *et al*. 1983).

Indirect-ELISA is limited to measurement of immunoglobulin classes present in low concentration (Kemeny & Challacombe 1988), due to high cost and the requirinment for highly skilled personnel (Miers *et al*. 1983; Thayer *et al*. 1987a). Nonetheless, the ELISA micro plate technique has shown to be highly sensitive, rapid and adaptable enough for studying many diseases affecting mammalian and avian species. Although the ELISA technique is becoming more popular, the HI test remains the conventional test for the detection and evaluation of antibodies against NDV (Snyder *et al*. 1983; Miers *et al.* 1983).

The HI test, as the conventional test, has been used to compare the sensitivity and specificity of the ELISA test and to measure the Kappa agreement between both tests. By definition (Brown *et al*. 1990), sensitivity is the capacity of the ELISA to be positive when the HI test is positive. Specificity is the capacity

of ELISA to be negative when the HI test is negative. Furthermore, Kappa agreement (Martin, Meek & Willeberg 1987; Brown et al. 1990) is defined as the proportion of the agreement beyond chance between two tests (The ELISA and HI test) in detecting antibodies to NDV.

Several studies to compare the relationship between ELISA and HI test for detection of antibody to NDV have been reported (Snyder *et al.* 1983; Miers *et al.* 1983; Marquardt *et al.* 1985; Thayer *et al.* 1987a,b; Adair *et al.* 1989; Brown *et al.* 1990; De Wit, Cvčlic-Čabrilo, Mazija, Bidin & Ragland 1992; Czifra *et al*. 1996, 1998).

Brown *et al*. (1990) reported 98.2 % of sensitivity and 91.7% specificity of ELISA-ND in comparison to the HI-ND test and a highly significant agreement (Kappa = 0.84, P<0.001) between two tests. In 1996, Czifra *el al.* reported 91.3 % of sensitivity, 76 % of specificity of ELISA-NDV and a good agreement (K = 0.67) between the two tests.

Difference between experimental conditions in comparing the ELISA-NDV and HI-NDV tests contributed to the discrepancies between the results among authors (Marquardt *et al.* 1985; Adair *et al.* 1989; Brown *et al*. 1990; Cvelić-Čabril Mazija, Bidin & Ragland 1992; Czifra *et al.* 1998).

The objectives of the present research project are the following to:

1. Provide further information on the protection induced by ND V4 vaccine to challenge by B1172 NDV isolated in South Africa in 1993 and
2. Compare the ELISA-NDV test and HI-NDV test in monitoring the response of NDV antibody induced during this experiment.

CHAPTER 3: MATERIAL AND METHODS

3.1. Experimental animals

Specific pathogen free (SPF) chickens and SPF embryonated eggs used in this experiment were obtained from Avimune[2], a commercial breeder of SPF White Leghorn. The breeder flock was certified for SPF diseases indicated in Table 3.1.

One hundred and four, day-old SPF White Leghorn chickens were obtained from Avimune and transported to the isolation unit of the Poultry Health Section, Department of Animal Studies at the Faculty of Veterinary Science. Chickens were randomly divided into groups of 16 to 18 and housed in wire-floored isolation units until 21 days old.

3.2. Housing

The isolation units were wire-floored isolators measuring approximately 1 m x 1 m x 1.5 m each (Fig 3.1). They were divided into two compartments, one used for rearing experimental chickens and other for disinfecting incoming and outgoing materials.

[2] Avimune (PTY) LTD, PO Box 14167 Centurion 0140, South Africa

Table 3.1. Certificated SPF diseases in the Avimune's breeder flock

Disease tested for	Sampling Date	Test	Result
Chicken Anemia Agent	30.01.1996	ELISA	Neg.
EDS- Infection	30.01.1996	ELISA	Neg.
Avian-Reo-Viruses	30.01.1996	ELISA	Neg.
Fowl Pox	30.01.1996	CE/PM	Neg.
Infectious Bronchitis	30.01.1996	ELISA	Neg.
Infectious Bursitis (IBD)	30.01.1996	ELISA	Neg.
Infectious Laryngotracheitis	30.01.1996	ELISA	Neg.
Influenza Type A-Infection	30.01.1996	ELISA	Neg.
Mycoplasma gallysepticum Infection	30.01.1996	Aggl	Neg.
Mycoplasma synovae Infection	30.01.1996	Aggl	Neg.
Newcastle Disease	30.01.1996	ELISA	Neg.
Salmonella pullorum Infection	30.01.1996	Aggl	Neg.
Salmonella enteridis Infection	30.01.1996	ELISA	Neg.
Turkey Rhinotracheitis (TRT)	30.01.1996	ELISA	Neg.
Ornithobacterium rinotracheale	30.01.1996	ELISA	Neg.
Infectious Coryza	30.01.1996	ELISA	Neg.

Neg. = Negative.
CE/PM = Clinical examination/post mortem.
AGP = Agar-Gel-Precipitation test.
Aggl = Agglutination test

One feeder and one automatic nipple drinker was installed in the first (rearing) compartment.

Figure 3.1. External view of the isolator units.

The environment within the isolators was controlled as follows:

3.2.1. *Laminar flow*

Laminar flow air with a positive pressure was supplied by an automatic system tested in accordance with U.S. Feed-std-290E for class 100 isolators. The air was pre-filtered and heated before entering the isolators.

3.2.2. Ventilation

The ventilation was set to supply 0.01 % of constant air change, 2 m/second of airflow, CO_2 less than 0.2 % and NH_3 less than 15 ppm. The humidity was fixed at 65-70 % within the isolation units by adjusting the temperature within the isolators.

3.2.3. Temperature

Temperature was regulated as stated in Table 3.2 to provide an optimum environment for growing replacement pullets.

Table 3.2. Temperature Adjustment within the isolators made throughout the experiment

Days-old	Temperature
1-7	30 °C ± 2 °C
8-14	28 °C ± 2 °C
15-21	24 °C ± 2 °C
> 21	21 °C ± 2 °C

3.2.4. Lighting

Artificial light exposure was manually controlled such that chickens of one to seven days of age were exposed to 24/24 hours light. Once the chickens were older than seven days of age they were exposed to 14/24 hours light up to the end of the experiment.

3.2.5. Stocking density

The stocking density per isolation unit was set according to the Code of Practice for the South Africa Poultry Association (SAPA). The stocking density used in this experiment was 14-16 chickens/m^2.

3.2.6. Feeding

The chickens were provided with a starter commercial ration and potable water *ad libitum*. The ration was obtained from SILGRO[3], provided in 50 Kg bags. During the experiment, the bags were stored on a table, placed within the experimental unit. They were kept covered with a plastic sheet to prevent any external contamination.

[3] SILGRO VOERE, Silverton 0127, Phone (012) 803-666, Pretoria, S.A.

3.3. Biosecurity

Entry to the experimental unit was restricted to authorized staff members only. Standard aseptic measures were used to ensure biosecurity

The experimental unit, isolator units and all equipment used in this experiment were disinfected using 2% Rectosept N (a glutaraldehyde combination disinfectant) solution obtained from a commercial supplier[4].

The experimental unit was disinfected (terminal disinfection) by spray method shortly before the experimental chickens were introduced. Approximately six hours after terminal disinfection, swabs were collected from the internal walls of the experimental unit, internal surfaces of the isolator units, feeders, drinkers, chicken boxes and from the table set within the experimental unit. The swabs were made by plate contact method using Petri dishes with an enriched agar media (APH Hunipath company, Hampshire England) and submitted for bacteriological counting at the Poultry Section. Standard techniques (Purchase, Dormermuth & Pearson 1989, Quinn, Carter, Markey & Carter 1994) were adopted for culturing and bacteriological counts. The results obtained were compared to the Microbiological Standards laid down by the South African Bureau of Standards (SABS).

The following additional measures for biosecurity were taken:

a) All equipment used in the experimental unit including disposable materials was transported to and from the experimental unit in sealed plastic bags. The bags were disinfected externally before leaving the experimental unit;

b) The feeders were filled twice a day (early morning and late afternoon) using disposable sealed plastic of 2-3 kg bags. The plastic bags were externally disinfected every time they entered the biosecurity cabinet. The first time at filling of the feeders and the second time when they were taken out;

c) Spray disinfection was always performed shortly after any treatment was conducted at the experimental unit, such as feeding, vaccinations or cleaning,

d) For all treatments and activities, control groups were handled before vaccinated groups.

3.4. Vaccination

3.4.1. Vaccination method

At 21 days of age, the surviving chickens were individually wing-tagged and randomly divided into three groups (A, B and C). The route of vaccination of each group is shown in Table 3.3.

[4] IMMUNO-VET Services P.O. Box 1825, Honeydew 2040, 9/11 South Africa.

Table 3.3. Classification of the groups

Group	Subgroup	Number of chickens	Method of Vaccination
Group A	Subgroup A1	7	Eye-drop
Group A	Subgroup A2	7	Eye-drop
Group A	Subgroup A3	7	Eye-drop
Group A	Subgroup A4	7	Eye-drop
Total		**28**	**Eye-drop**
Group B	Subgroup B1	7	in-contact
Group B	Subgroup B2	7	in-contact
Group B	Subgroup B3	7	in-contact
Group B	Subgroup B4	8	in-contact
Total		**29**	**in-contact**
Group C	Subgroup C1	16	Control
Group C	Subgroup C2	14	Control
Total		**30**	**Control**

Group's A and B were each randomly divided into four sub-groups designated A1, A2, A3, A4, and B1, B2, B3 and B4 respectively. Group C was randomly divided into two sub-groups designated groups C1 and C2 (Table 3.3). The allocation of the different groups into specific isolator units is shown in Table 3.4. and Figure 3.2.

The unvaccinated in-contact chickens (Groups B1-B4) were able to mingle in the same isolation unit with an equal number of vaccinated chickens (Groups A1-A4). Therefore, group A1 was combined with group B1 in the isolation unit 1, group A2 was combined with group B2 in unit 4, group A3 with group B3 in isolation unit 6, and group A4 with group B4 in unit 7. Group's C1 and C2 were placed into isolators 3 and 8 respectively (Table 3.4).

Table 3.4. Placement of chickens in the isolator units

Isolator unit number	Subgroup of eye-drop vaccinated chickens	Subgroup of in-contact chickens	Unvaccinated control	Total of birds per isolator
1	A1	B1	-	14
4	A2	B2	-	14
6	A3	B3	-	14
7	A4	B4	-	15
3	-	-	C1	16
8	-	-	C2	14
Total				**87**

Eye-drop vaccinations were performed on days 21 and 35 of age as recommended by Spradbrow (1987).

3.4.2. Reconstitution and use of the ND V4 vaccine

Freeze-dried ND V4 vaccine was reconstituted with Webster's vaccine diluent on days 21 and 35. Freeze-dried ND V4 vaccine was reconstituted one hour before vaccination took place. The Webster's vaccine diluent was prepared at Poultry Reference Laboratory following the prescription provided by the Arthur Webster Company[5]. One vial of ND V4 vaccine was mixed with 40 mL of Webster's vaccine diluent and provided 1000 doses with 10^6 EID_{50}/mL (Mean Embryo infectious doses/mL), to be given by eye-drop route. The bulk of the vaccine was bottled in 2-mL plastic vials capped with eye-drop applicators. The plastic vial and eye-drop applicators were obtained from Avimune.

[5] Arthur Webster Pty Limited. P.O Box 234, NSW 2153, Australi

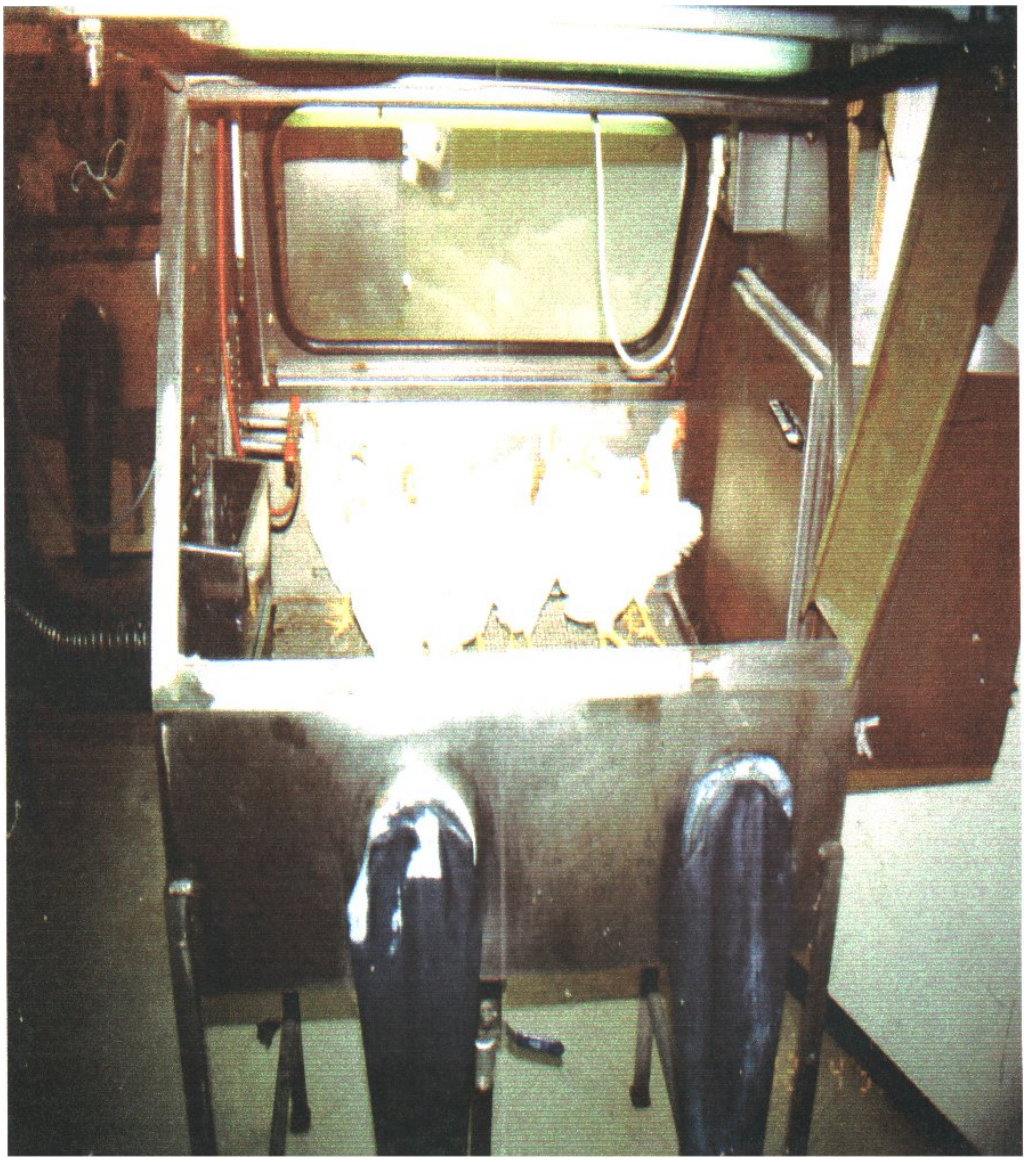

Figure 3.2. Internal view of the Poultry section's isolator units

One plastic vial containing only Webster's diluent was prepared for the placebo vaccination of control groups. One bird dose was approximately 0.04 mL (2

drops) applied into the eye. A placebo vaccine was administrated in 50% of the chickens in the unvaccinated control groups.

On each occasion, the vaccination was completed within three hours after the vaccine was reconstituted. Samples of reconstituted vaccine were collected one hour before and immediately after vaccination for the determination of the HA titres (See section 3.5).

3.5. Quality control of Vaccine

Vials of freeze-dried ND V4 vaccine (Batch Number 91740) and the prescription of the diluent of the ND V4 vaccine were all provided by the Arthur Webster's Company. Each vial contained 1000 doses with 10^6 EID_{50}/mL.

One freeze-dried vial of ND V4 vaccine was taken and tested for haemagglutinating activity (HA) and titrated in SPF embryonated chicken eggs for infectivity. The HA assay was performed in duplicate following the method described by Allan & Gough (1974) and Beard (1989). The HA assays were conducted using V-well microtiter plates, reconstituted ND V4 vaccine, 0.3 % chicken erythrocytes, known control serum (standard positive and negative serum) and 2 % Veronal buffer mixed in PBS. The 100 % solution of Veronal buffer was supplied by the Poultry Reference Laboratory of the Section of Poultry Health, in the Department of Production Animal Studies, Faculty of Veterinary Science. Briefly, 50 µl of 2 % Veronal buffer was placed in all well/row of the microtiter plate. Thereafter, 50 µl of reconstituted ND V4 vaccine was mixed in the

first well/row of the microtiter plate to make up a 2^{-1} dilution. Two-fold serial dilutions were made across the plate up to 2^{-12} dilution. Chicken erythrocytes suspension of 0.3% was added to all wells including the controls. The microplates were then incubated at room temperature for 45 minutes.

A positive haemagglutination of erythrocytes (HA^{+}) was taken as the presence of haemagglutination (hazy film of erythrocytes) and the negative (HA^{-}) as the absence of haemagglutintion (sharp button of erythrocytes) observed on the V-bottom microtiter plates. The HA titer was interpreted as the reciprocal of the highest dilution of serum at which 100 % positive erythrocytes haemagglutination resulted. The identity of the ND V4 vaccine as NDV was then confirmed by HI test (see 3.11 serology).

The titration of the ND `V4 was performed in 10-day old SPF embryonated chick eggs as described by Villegas & Purchase (1989). Briefly, 9.0 mL of 2 % Veronal buffer was placed in all wells/row of a microtiter plate. Thereafter, one mL of the reconstituted vaccine was taken and used to perform 10-fold serial dilution up to 10^{-10}. Then, 0.1 mL of each dilution (from 10^{-2} to 10^{-10}) was inoculated into each of five 10 day-old SPF embryonated chick eggs and incubated at 37 ± 2^{0} C for 6 days. The eggs were examined daily and each embryo scored as dead or alive. Mortalities that occurred within 24 hours post-inoculation were considered to be non-specific and were not included in the results. All dead or dying embryos found during the 6 days period of observation were chilled at 4^{0} C for 3 hours and the allantoic fluid harvested and stored individually. Thereafter, all allantoic fluids were tested for NDV HA as described previously.

The quantal response of the ND V4V in embryonated eggs was calculated from the proportion of dead embryos that were inoculated with a series of 10-fold dilutions (Villegas and Purchase (1989) together with the HA results as described by Spradbrow & Samuel (1992). Afterwards, the 50 % end point was determined according the method of Reed and Muench (1938). The titer of the infectivity of the ND V4V vaccine was interpreted as the reciprocal exponential of the 50 % end point dilution and expressed as EID_{50}.

3.6. Challenge Virus and method

The challenge virus was the freeze-dried B1172/93 South African field isolates of NDV made from the 1973 outbreak that occurred in South Africa (Coetzee, personal-communication 1993). Aliquots of 0.30 mL freeze-dried B1172/93 NDV were supplied by the Poultry Reference Laboratory. These had been prepared by propagating the B1172 virus, a field isolate of NDV, in the allantoic fluid of 9-11 day old SPF embryonated eggs. The B1172 NDV was tested for HA activity and its identity confirmed using the HI test (with known positive NDV antiserum). The allantoic fluids with high titer of B1172/93 NDV were mixed with equal volume of buffer lactose peptone (BLP)[6], before being freeze-dried and stored at -4 °C.

The B1172 NDV was tested twice for HA and titrated for infectivity in SPF embryonated chicken eggs. On each occasion, one aliquot of the freeze-dried B1172 NDV was reconstituted with one mL of sterile PBS and the HA and

[6] Onderstepoort Biological Products. P/Bag x04 Onderstepoort 0110. Pretoria. South Africa

infectivity titres were determined using the same procedures described for the ND V4V. The titer of the B1172 NDV was taken as the mean calculated from the titrations and expressed in EID_{50}.

The challenge dose/bird was estimated from the infectivity titer to contain $1x10^2$ EID_{50} per mL. The original titer with $10^{8.8}$ EID_{50}/mL of the B1172 NDV was converted into a challenge dose of $1x10^2$ EID_{50} per mL applying the method of logarithms described by Villegas and Purchase (1989). Briefly, the challenge solutions were prepared as a 1/10 dilution of one aliquot of freeze-dried B1172/93 NDV with sterile PBS. Thereafter, 1 mL was taken to perform ten-fold serial dilutions up to 10^{-6} dilution. The challenge solutions with $10^{6.3}$ EID_{50} per mL of B1172 NDV isolate were prepared by mixing 4 mL of 10^{-6} dilution with 21.2 mL of PBS to obtain a final volume of 25.2 mL.

At 49 days of age, all the chickens were challenged via the intratracheal route. The challenge dose per chicken was 0.2 mL of $10^{6.3}$ EID_{50} per mL of B1172 NDV. A tuberculin syringe fitted with a 50 mm 16 gauge blunt needle was used to deliver the challenge solution into the anterior area of the trachea (Coetzee 1980).

Samples of challenge solution were taken shortly after preparation and the HA titer determined by the methods described above. The identity of the B1172 NDV was also confirmed by HI test.

3.7. Clinical signs

All chickens were examined twice a day for clinical signs (early morning and late afternoon). A dichotomous scoring system was used to record the clinical signs: "0" if a chicken was found normal and "1" if it was showing clinical signs of dyspnea, nasal discharge, conjunctivitis, listlessness, prostration and/or nervous signs.

Mortalities were recorded as they occurred during the experiment. Dead birds were removed from the isolator unit as soon as they were discovered. On the 15th day after challenge, all surviving birds were euthanased by cervical dislocation (Zander & Mallisson 1991).

Necropsies were carried out on all birds that died during the experiment and on those sacrificed at day 63. All the necropsies were performed within the experimental unit. The value "0" was used when no gross lesions were observed and "1" if there was evidence of haemorrhagic lesions in the respiratory or gastrointestinal tract, or in a particular organ or tissue. Trachea, lung, proventriculus and caecal tonsils were collected and placed into individual containers for each chicken and submitted to the Poultry Health Department for virus and bacterial isolations.

3.8. Virus isolation

Tissue samples were suspended in PBS containing antibiotics (Penicillin and Streptomycin) and immediately stored at -20 until they were required for virus isolation.

Tests for NDV isolation were carried out at the Poultry Reference Laboratory (PRL) at section of Poultry Health, in the Department of animal Studies, Faculty of Veterinary Science, University of Pretoria.

Tissue samples were subjected to three passages before they were regarded as negative for NDV isolation. Samples that were positive to NDV isolation were selected and submitted to CVL-Weybridge, England, for a comparative characterization of the identity of the recovered ND virus.

3.9. Bacterial Examination

Tissue samples were placed on Petri dishes and submitted to the PRL, for bacteriological examination.

3.10. Serology

B-HI test and indirect-ELISA were used to monitor the antibody response to NDV induced throughout the experiment.

3.10.1. Haemagglutination inhibition (HI) test

The *B*-HI NDV test was performed as described by Beard (1989). The HI assays were conducted in V- bottom microtiter plates using 4 HA units of inactivated *La Sota* strain of NDV per well, 3 % of chicken erythrocytes, blood serum samples and recognized control serum (standard positive and negative serum to NDV antibodies).

A) PREPARATION OF THE *LA SOTA* ANTIGEN

The *La Sota* antigen was provided by the PRL. The antigen had been produced by inoculating 0.2 mL of 10^{-3} dilution of PBS, saturated with antibiotics (Penicillin and streptomycin) into the allantoic fluid of five 9 day-old SPF embryonated eggs. When approximately 20% of embryos had died, the allantoic fluid was harvested and pooled and the HA test was performed on the allantoic fluid.

Treating the infective allantoic fluid with 0.1% formalin carried out the inactivation of the *La Sota* NDV. This solution was kept overnight at room temperature. On the following day, the inactivation was tested by inoculation of SPF eggs. The allantoic fluid containing inactivated *La Sota* NDV was divided into aliquots of one mL each and stored at -10 .

A single aliquot of inactivated *La Sota* antigen was used for each HI test.

B) PREPARATION OF 0.3% CHICKEN ERYTHROCYTES SUSPENSION

Blood samples were collected by wing vein puncture from 7-8 months old White Leghorn layers free of ND antibodies (provided by the Poultry Reference Laboratory, Faculty of Veterinary Science/UP, Onderstepoort) into a syringe containing an equal volume of Alsever's solution (Sonnenwirth 1970) to prevent coagulation. Thereafter, blood was diluted 200 - fold with 2 % Veronal buffer and centrifuged at 2000 rpm for 5 minutes. Then, the supernatant was decanted and the packed erythrocytes were suspended again in 2% Veronal buffer and re-centrifuged. The decanting suspension and centrifugation were repeated for a third time. The packed erythrocytes were used to prepare a 0.3 % suspension based on volume by diluting 0.3 mL of packed erythrocytes with 99.7 mL of 2% Veronal buffer (pH 7.0- 7.2).

C) BLOOD SAMPLES

Blood samples were collected from each of the experimental chickens by wing vein puncture on days 21, 35, 49 and 63 of age. Thus, blood samples were collected before vaccination (at 21 and 35 days), at pre-challenge (at day 49) and before euthanasia (at day 63 of age) respectively. The blood samples were allowed to clot in 5 mL plastic tubes at room temperature (Alexander 1988) for 3 hours. After this, the clot was loosened and the tubes were stored at 4 overnight. Serum was removed on the following day. The serum was then tested for NDV antibodies using the HI and ELISA tests. The remaining serum was stored at -20 ^{o}C at the Poultry Reference Laboratory.

The HI tests were interpreted as the reciprocal of the highest dilution of serum at which 100% inhibition of haemagglution was observed. The results were expressed as the indices of Log_2.

3.10.2. Seroconversion

Seroconversion scores were estimated from the results of the NDV- HI titer. All chickens with a Log_2 HI antibody titer equal and higher than 4 value were scored as positive Seroconversion (Brown *et al.* 1990, Alexander 1991). All chickens with a Log_2 HI antibody titer less than 4 were scored as negative seroconversion.

3.10.3. ELISA test

A) REAGENT PREPARATION

The ELISA test conducted in this experiment used semi-automatic equipment and Delta Byproduct ND ELISA kit purchased from Sterilab[7]. The ELISA kit was composed of NDV antigen U-bottom well plates, washer buffer at 20 x concentrate, 10 x conjugate, conjugate diluent and standard controls. The ELISA equipment included a microplate reader and printer, Delta wash-system and manifold, Tray shaker, 8-channel micropippete, Sharp EL5150 calculator and timer and other disposable materials.

All reagents were prepared according to the recommendations of the Delta Bioproducts company.

B) ELISA TEST PROCEDURE

The ELISA-NDV was performed following the procedures recommended by Sterilab as well as a modified indirect-ELISA described by Snyder *et al*. (1983). Briefly, serum samples and standard controls were pre-diluted on U-bottom dilution microplate at 1:40 using 1 x wash buffer. A microplate coated with NDV antigen (testing microplate) was rinsed twice and tap dried on absorbent paper. 100 µl of serum and standard controls were added into specific marked wells of microplate. The microplate was incubated for 15 minutes on a plateshaker at room temperature and washed as described previously. 100 µl of 1 x conjugate was distributed into each well of the testing microplate. The test microplate was incubated and washed as described previously. A volume of 100 µl of substrate was added into each well and incubated for 15 minutes as described previously. Finally, 200 µl of stop reaction was transferred into each.

The optical density of the ELISA-NDV was recorded using an EL307 C microplate spectrophotometer reader set at 550 nanometers. The spectrophotometer reader was previously calibrated on an unsensitized U-well microplate containing 100 µl of substrate reaction and 100 µl of the stop reaction.

[7] Sterilab services, P.O Box 2021, Kempton Park 1621, Johannesburg, South Africa

The optical density values and standard index values were used to determine the linear regression coefficient (*r*). Corrected index values were calculated from *r* and these used to estimate the relative titer of ELISA-NDV antibody (STERILAB, 1996 private communication). The ELISA-NDV antibody titer given on Log_{10} N then converted to Log_2 N as described by Boyle and Cunninghan (1989).

The ELISA-NDV test results were expressed as optical density values and index observance values. The ELISA NDV-antibody titer expressed as Log_2. Any results with *r*-value less than 0.95 was considered invalid in this experiment.

3.11. Protection conferred by ND V4 against B1172 NDV

Protection was defined as the ability of chickens to survive challenge with B1172 NDV isolate, with or without clinical signs or gross lesions.

3.12. Correlation between HI test and ELISA test for the detection of antibody to ND

The Delta commercial NDV-ELISA is designed to be positive when the index value is at or greater than 0.8 while the corresponding HI-titer is $Log_2 \geq 6$. Therefore, all index values less than 0.80 or $Log_2 < 6$ HI-titer were considered negative for ELISA- NDV antibody response. All index values greater than 0.80 or $Log_2 \geq 6$ HI-titer were considered positive for NDV antibody response.

The relative sensitivity and relative specificity of ELISA-NDV and the agreement (Kappa) between ELISA-NDV and HI-NDV test were calculated according to the procedures described by Martin *et al.* (1987).

3.13. Statistics

Data were individually recorded and processed for statistical analyses using the EPINFO[8] software program. The data were used to analyse sensitivity and specificity values of ELISA-NDV against HI test and the agreement beyond chance (K agreement) between two tests.

The following levels of K agreement were used in the interpretation of agreement between the ELISA-NDV and HI-NDV test:

a) Kappa values < 0.40 were taken to represent no agreement;
b) Kappa values of o.40 – 0.70 were taken to represent moderate agreement and
c) Kappa values of ≥ 0.70 were taken as a good agreement.

Student- t test, Chi-square test and Fischer exact test were used for statistical analysis. All results with P> 0.05 were taken as not statistically significant.

[8] Centres for Disease Control and Prevention. Mailstop C08 EPO. Atlanta, GA 30333. U.S.A.

CHAPTER 4: RESULTS

4.1. Experimental animals

Of the one hundred and four one-day-old, white Leghorn chickens obtained, 17 had died by the time they were 21 days old. Five of these were culled and sacrificed (four because they showed clinical signs of toe paralysis and one due to conjunctivitis). Twelve chickens died of heat stress due to thermostat failure in isolator unit 7 at 10 days old.

Behaviour characterized by nervousness (fright), and scratching out ration from the feeder was evident in growing chickens from day 35. This behaviour was more intensive as the birds were growing.

4.2. Housing

Tables 4.1a, 4.1b show the temperature recorded within the cage units.

Table 4.1a. Temperature recorded during the experiment

Age*	Temperature in			
	Unit1	Unit2	unit 3	unit 4
	Mean ± SD		Mean ± SD	Mean ± SD
1-7	29 ±0.6	29 ± 0.6	29 ± 0.6	29 ± 0.6
8-14	26 ± 0.9	26 ± 0.9	26 ± 0.9	26 ± 0.9
15-21	24 ± 0.6	24 ± 0.5	24 ± 0.5	24 ± 0.5
22-63	21 ± 0.6	-	22 ± 0.8	21 ± 0.5

*: Days old **: Unit 2 was used only during the rearing period
SD: Standard deviation:

Table 4.1b. Temperature recorded during the experiment

Age*	Temperature in		
	unit 6	unit 7	unit 8
	Mean ± SD	Mean ± SD	Mean ± SD
1-7	29 ± 0.6	29 ± 0.6	29 ± 0.6
8-14	26 ± 0.9	30 ± 11	26 ± 0.9
15-21	24 ± 0.5	22 ± 0.5	24 ± 0.5
22-63	21 ± 0.6	22 ± 0.6	22± 0.6

*: Days old **: Unit 2 was used only during the rearing period
SD: Standard deviation:

A temperature of 55 °C, caused by a failure of the thermostat, was recorded in isolator unit 7 on day 10 of age.

The floors of the isolator units were occluded twice during the experiment with scratched ration and the litter. For biosecurity (see section 4.3), all isolators units including the experimental unit were cleaned twice during the experiment.

4.3 Biosecurity

A high level of bacterial contamination was seen within the experimental unit and isolators 1 and 8 (Table 4.2.) before the day of arrival of the experimental animals. Thus, a second disinfection was carried out in all isolator units and the experimental unit shortly before the experimental animals had been allocated.

Table 4.2. Results of bacterial counts (in CFU/10 cm^2) taken after sanitation of the experimental units and isolator units

	Position within the isolator unit					
Place of Swabbing	Feeder	Left	Right	Floor	Roof	Average
Isolator 1	7.51	1.73	2.31	144.5	1.15	31.4
Isolator 3	22	2.31	0.6	1.7	10.4	7.4
Isolator 4	0.6	0.02	1.15	1.73	0	0.35
Isolator 6	TNTC	0.02	1.7	0	0.57	0.57
Isolator 7	0	4.05	14.45	2.89	1.16	4.51
Isolator 8	0.6	0	4.6	10.4	1.15	3.04
On the Table	-	-	-	57.8	ER	57.8
Experimental unit	-	-	57.8*	ER	ER	57.8

TNTC: To numerous to count ER: Excluding results
*: Internal walls of the experimental unit

As measures for biosecurity from environmental stress within the isolators units (See section 4.1 and 4.2), the isolators unit 3 and 8 were cleaned out at day 29 of age and repeated at 41 days old. Isolators unit 1, 2, 4 and 7 were cleaned at 36 days of age and again at day 42.

4.4. **Vaccination**

All chickens remained healthy during the period between the two vaccinations and for 14 days following the second vaccination.

4.5. Quality Control of Vaccine

One unused vial of the ND V4 vaccine was tested for haemagglutination activity (HA) and titrated for infectivity in 9-10 day-old embryonated SPF eggs. Results are shown in Tables 4.3 and 4.4.

Table 4.3. Results of titration of the ND V4 vaccine in 9 to 11 day-old SPF embryonated eggs

a) Determination of the 50 % end point based on the embryonic death

Virus dilution	Inoculated Embryos		Cumulative numbers		Proportion	%
inoculated	Dead	Alive	Dead	Alive	Dead/Total	Dead
10^{-1}	0	4	9	4	9/13	69
10^{-2}	2	3	9	7	9/16	56
10^{-3}	2	2	7	9	7/16	43
10^{-4}	1	3	5	12	5/17	29
10^{-5}	1	3	4	15	4/19	21
10^{-6}	1	3	3	18	3/21	14
10^{-7}	2	2	2	20	2/22	9
10^{-8}	0	4	0	24	0/24	0
10^{-9}	0	4	0	28	0/28	0
10^{-10}	0	4	0	32	0/32	0
Control	0	4	0	4	0/32	0

b) Determination of the 50 % end point based on the presence of NDV haemagglutins in the allantoic fluid

Virus dilution	Allantoic fluid*		Cumulative Numbers		Proportion	%
inoculated	HA^+	HA^-	HA^+	HA^-	HA^+/Total	HA^+
10^{-1}	4	0	26	0	26/26	100
10^{-2}	5	0	22	0	22/22	100
10^{-3}	4	0	17	0	17/17	100
10^{-4}	4	0	13	0	13/13	100
10^{-5}	4	0	9	0	9/0	100
10^{-6}	3	0	5	1	5/6	83
10^{-7}	2	2	2	3	2/5	40
10^{-8}	0	4	0	7	0/7	0
10^{-9}	0	4	0	11	0/11	0
10^{-10}	0	4	0	15	0/15	0
Control	0	4	0	4	0/4	0

*: Allantoic fluid harvested from all inoculated eggs
HA^+: Presence of NDV haemagglutinins
HA^-: Absence of NDV haemaglutinins

Table 4.4. Titer of HA and EID_{50} and the Mean Death Time (MDT) of the ND V4 vaccine

Newcastle Disease virus strain	HA Titer (Log2)	EID_{50}	MDT (Hours)
Freeze-dried V4 vaccine	3	$10^{8.23}$	>168[a]
Reconstituted V4 vaccine[b] at 21 days	3	ND	ND
Reconstituted V4 vaccine[c] at 21 days	4	ND	ND
Reconstituted V4 vaccine[b] at 35 days	3	ND	ND
Reconstituted V4 vaccine[c] at 35 days	4	ND	ND

a: After egg inoculation.
b: Samples collected shortly after reconstitution of the vaccine and shortly before vaccination
C: Samples collected shortly after vaccination
ND: Not done

The V4 ND vaccine gave an average $10^{7.2}$ EID_{50} (Table 4.3b). The V4 NDV vaccine did not cause mortality in the embryonated eggs during 7 days after inoculation

4.6. Challenge and method

The freeze dried B1172 NDV was titrated twice for HA and for infectivity in 10 day-old embryonated SPF eggs. Results are given in Tables 4.5 and 4.6.

The freeze-dried B1172 NDV killed 100% of inoculated embryos within 48 hours. The highest 10-fold dilution that killed 100 % of embryo was at 10^{-6} dilution on the first titration and at 10^{-7} dilutions on the second titration.

Mortality was recorded after challenge and can be seen in Table 4.7.

Table 4.5. Results of titration of the B1172 challenge NDV in 9 to 11 day-old SPF embryonated eggs

a) First titration

Virus dilution	Inoculated Embryos		Cumulative numbers		Proportion	%
Inoculated	Dead	Alive	Dead	Alive	Dead/Total	Dead
10^{-1}	5	0	39	0	39/39	100
10^{-2}	5	0	34	0	34734	100
10^{-3}	5	0	29	0	29/29	100
10^{-4}	5	0	24	0	24/24	100
10^{-5}	5	0	19	0	19/19	100
10^{-6}	5	0	14	0	14/14	100
10^{-7}	5	0	9	0	9/9	100
10^{-8}	1	4	4	4	4/8	50
10^{-9}	2	3	3	7	3/10	30
10^{-10}	0	5	1	12	1/13	7
10^{-11}	1	4	1	16	1/17	6
Control	0	5	0	5	0/5	0

b)Second titration

Virus dilution	Embryos		Cumulative numbers		Proportion	%
inoculated	Dead	Alive	Dead	Alive	Dead/Total	Dead
10^{-1}	5	0	36	0	36/36	100
10^{-2}	5	0	31	0	31/31	100
10^{-3}	5	0	26	0	26/26	100
10^{-4}	5	0	21	0	21721	100
10^{-5}	5	0	16	0	16/16	100
10^{-6}	5	0	11	0	11/11	100
10^{-7}	4	1	6	1	6/7	86
10^{-8}	1	4	2	5	2/7	29
10^{-9}	1	4	1	9	1/10	10
10^{-10}	0	5	0	14	0/14	0
10^{-11}	0	5	0	19	0/19	0
Control	0	2	0	2	0/2	0

Table 4.6. Results of HA, EID_{50} and the Mean death time (MDT) of the B1172 challenge NDV

Titration	EID_{50}	HA titer (Log2)	MDT (Hours)
First titration	8.0	8	48
Second titration	7.6	8	72
Mean	7.8	8	60

Table 4.7. Results of challenge with B1172 NDV

a) Chickens vaccinated by Eye-drop method

	Number of chickens			
Group	Challenged	Died	Survived	survival
A1	7	0	7	100
A2	7	0	7	100
A3	7	0	7	100
A4	7	0	7	100
Total	**28**	**0**	**28**	**100**

b) Chickens vaccinated by in-contact method

	Number of chickens			%
Group	Challenged	Died	Survived	survival
B1	7	4	3	43
B2	7	6	1	14
B3	7	3	4	57
B4	8	0	8	100
Total	**29**	**13**	**16**	**55**

c) Unvaccinated control chickens

	Number of chickens			%
Group	Challenged	Died	Survived	survival
C1	16	5	11	68
C2	14	7	7	50
Total	**30**	**12**	**18**	**60**

4.7. Clinical Signs

The signs of disease appeared 6 days following challenge. The clinical signs were characterized by a progressive depression following dispnoea, nasal discharge of muco-fibrinous fluid and listlessness. A whitish - green diarrhoea and nervous signs (torticollis) were seen in two chickens in the unvaccinated control groups. By the 3rd-4th day of illness, the birds showed a progressive inappetence to a complete starvation. Mortality started occurring from 9th - 10th after challenge up to day 63 (14 days after challenge). Figure 1 shows chickens exhibiting signs of disease.

Table 4.8 and 4.9 summarize the clinical signs and gross lesions shown by birds after challenge with B1172 NDV.

Clinical signs were observed in 7/28 (25 %), 14/29 (48 %) and 13/30 (43 %) chickens in-group A, B, and C respectively.

All chickens that died in the groups B and C showed clinical signs. In the surviving chickens, clinical signs were seen in 7/28 (25 %), 1/16 (6 %) and 1/18 (6 %) of chickens in the groups A, B and C respectively.

In the respiratory tract, a moderate to a severe mucoid-fluid discharge and diffuse petechial haemorrhages on the trachea were observed (Figure 4.2). About 23 % (20/87) of chickens at necropsy showed an extensive lung congestion and a moderate opacity of the air sacs (20/8). In the

gastrointestinal tract, petechial hemorrhages were seen on the caecal tonsils. Splenomegaly was observed in most vaccinated chickens.

Figure 4.1. Sick chickens

Gross lesions in the absence of clinical signs were observed in some birds in all groups.

Table 4.8. Numbers of dead birds showing clinical signs, gross lesions after challenge with B1172 NDV

a) Chickens vaccinated by eye-drop method

Group	Total of dead birds	Clinical signs		Respiratory tract lesions		Gastrointestinal lesions	
		No	Yes	No	Yes	No	Yes
A1	0	0	0	0	0	0	0
A2	0	0	0	0	0	0	0
A3	0	0	0	0	0	0	0
A4	0	0	0	0	0	0	0
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**

b) Chickens vaccinated by in-contact method

Group	Total of dead birds	Clinical signs		Respiratory tract lesions		Gastrointestinal lesions	
		No	Yes	No	Yes	No	Yes
B1	4	0	4	1	3	3	1
B2	6	0	6	0	6	5	1
B3	3	0	3	0	3	3	0
B4	0	0	0	0	0	0	0
Total	**13**	**0**	**13**	**1**	**12**	**11**	**2**

c) Unvaccinated Control chickens

Group	Number of dead birds	Clinical signs		Respiratory tract lesions		Gastrointestinal lesions	
		No	Yes	No	Yes	No	Yes
B1	5	0	5	1	4	2	3
B2	7	0	7	0	7	4	3
Total	**12**	**0**	**12**	**1**	**11**	**6**	**6**

Table 4.9. Number of sacrificed birds showing clinical signs and gross lesions after challenge with B1172 NDV

a) Chickens vaccinated by eye-drop method

Group	Number of dead birds	Clinical signs		Respiratory tract lesions		Gastrointestinal lesions	
		No	Yes	No	Yes	No	Yes
A1	7	4	3	4	3	4	3
A2	7	6	1	2	5	5	2
A3	7	4	3	2	5	7	0
A4	7	7	0	5	2	7	0
Total	**28**	**21**	**7**	**13**	**15**	**23**	**5**

b) Chickens vaccinated by in-contact method

Group	Number of dead birds	Clinical signs		Respiratory tract lesions		Gastrointestinal lesions	
		No	Yes	No	Yes	No	Yes
B1	3	3	0	2	1	3	0
B2	1	1	0	0	1	1	0
B3	4	3	1	2	2	4	0
B4	8	8	0	6	2	6	2
Total	**16**	**15**	**1**	**10**	**6**	**14**	**2**

c) Unvaccinated Control chickens

Group	Number of dead Birds	Clinical signs		Respiratory tract lesions		Gastrointestinal lesions	
		No	Yes	No	Yes	No	Yes
C1	11	10	1	2	9	4	7
C2	7	7	0	1	6	7	0
Total	**18**	**17**	**1**	**3**	**15**	**11**	**7**

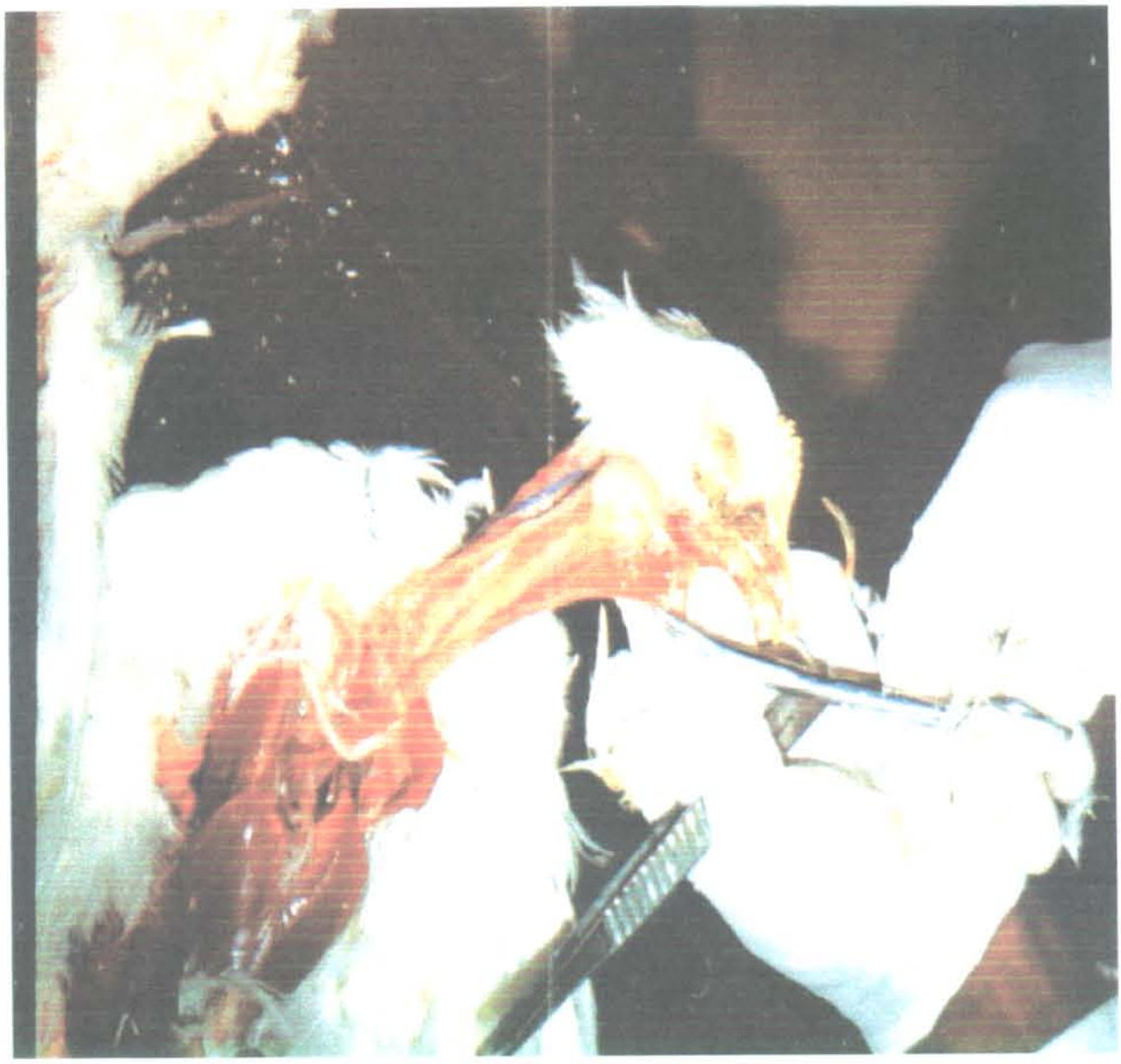

Figure 4.2. Trachea congested indicated by red spots in inner wall

Gross lesions in the respiratory tract were seen in 12/13 (92 %) and 11/12 (92 %) chickens that died after challenge in group B and C respectively. In surviving chickens, lesions in the respiratory tract were observed in 15/28 (54 %), 6/16 (37.5 %) and 15/18 (83 %) of chickens in the groups A, B and C respectively.

In dead chickens, gastrointestinal lesions were observed in 2/13 (15 %) chickens vaccinated by the in-contact method and in 6/12 (50 %) of the unvaccinated control birds. In surviving birds, gastrointestinal lesions were found in 5/28 (18 %) of the eye-drop vaccinated groups, 2/16 (12,5 %) of the in-contact vaccinated groups and 7/18 (39 %) in the unvaccinated control groups.

Lesions of the respiratory tract were more often observed than lesions of the gastro-intestinal tract.

4.8. NDV Isolation

The NDV re-isolation made from tissue samples collected at the necropsies is shown in Tables 4.10 and 4.11.

Table 4.10: Results of NDV isolations/ egg passages

a) Chickens vaccinated by eye-drop method

Group	First passage		Second passage		Third passage	
	No	Yes	No	Yes	No	Yes
A1	7	0	7	0	7	0
A2	7	0	7	0	7	0
A3	7	0	7	0	7	0
A4	7	0	7	0	7	0
Total	**28**	**0**	**28**	**0**	**28**	**0**

b) Chickens vaccinated by in-contact method

Group	First passage		Second passage		Third passage	
	No	Yes	No	Yes	No	Yes
B1	6	1	6	0	6	0
B2	3	4	3	0	2	1
B3	5	2	5	0	5	0
B4	8	0	8	0	8	0
Total	**22**	**7**	**22**	**0**	**21**	**1**

b) Unvaccinated Control chickens

Group	First passage		Second passage		Third passage	
	No	Yes	No	Yes	No	Yes
C1	14	10	4	0	14	0
C2	11	3	8	0	11	0
Total	**25**	**13**	**12**	**0**	**25**	**0**

About 95 % of the NDV isolations were made at the first egg passage and 5 % at the third egg passage.

Table 4.11: Results of NDV isolations made on dead and sacrificed birds after challenge with B1172 NDV

a) Chickens vaccinated by eye-drop method chickens

	Dead Pullets			Surviving Pullets		
Group	Total	No	Yes	Total	No	Yes
A1	0	0	0	7	7	0
A2	0	0	0	7	7	0
A3	0	0	0	7	7	0
A4	0	0	0	7	7	0
Total	**0**	**0**	**0**	**28**	**28**	**0**

b) Chickens vaccinated by in-contact method

	Dead Pullets			Surviving Pullets		
Group	Total	No	Yes	Total	No	Yes
B1	4	3	1	3	3	0
B2	6	1	5	1	1	0
B3	3	1	2	4	4	0
B4	0	0	0	8	8	0
Total	**13**	**5**	**8**	**16**	**16**	**0**

c) Unvaccinated control chickens

	Dead Pullets			Surviving Pullets		
Group	Total	No	Yes	Total	No	Yes
C1	5	3	2	11	3	8
C2	7	4	3	7	7	0
Total	**12**	**7**	**5**	**18**	**10**	**8**

NDV was re-isolated in 8/29 (27.5 %) of the in-contact groups and in 13/30 (43 %) of the control group. No NDV isolation was observed from eye-vaccinated chickens.

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NDV- isolations occurred in 8/13 (61.5 %) of group B and in 5/12 (41.6 %) of group C of chickens that died during the challenge. In surviving chickens, NDV isolations occurred only in 8/18 (44.4%) of unvaccinated control chickens. No

isolations of NDV were demonstrated in the group A and others birds in the group B that survived challenge.

4.9. Bacterial examination

Out of 87 tissue samples submitted for bacterial examination, 18 were positive for bacterial isolation. Bacterial isolations could be classified into the genera *Klebsiella, Enterobacter, Pseudomonas, Proteus, Staphyloccocus* and *Lactobacillus. Escherichia coli* was also isolated. Individual or mixed colonies of bacteria were isolated from some birds.

4.10. Serology

4.10.1. Antibody response to NDV

Tables 4.12, 4.13, 4.14 and 4.15 show the results of the mean HI-NDV antibody titer, the mean value of ELISA-NDV optical density (OD), ELISA index (IV) and ELISA-NDV related antibody titer.

No detectable antibody to NDV was observed in birds at 21 days of age by the HI test. A slight rising of HI –NDV titer (< Log_2 4) was recorded by day 35 (14 days after primary vaccination) and at day 49 (14 days after secondary vaccination) antibody titer in all groups. HI-NDV titres of Log_2 >4 were only recorded in the group A4 at day 35 and in the groups A4 and B4 at 49 days of age.

At 63 days of age, all survivors had an HI-NDV antibody titer equal or higher than Log2 4 in all chickens that survived challenge. The group A4 showed a lower HI-NDV titer compared with others groups of vaccinated chickens. In unvaccinated control chickens, the level of HI-NDV titer in the group C1 was lower compared with group C2.

Table 4.12. Mean HI-NDV antibody titres (Log_2) of pullets bled at 21, 35, 49 and 63 days of age

a) Eye-drop vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
A1	0.0 ± 0.0	1.0 ± 1.4	0.9 ± 1.2	11.7 ± 0.8
A2	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 1.2	12.0 ± 0.0
A3	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.8	10.1 ± 3.2
A4	0.0 ± 0.0	1.0 ± 2.7	2.9 ± 2.1	5.6 ± 3.0
Total	**0.0 ± 0.0**	**0.4 ± 1.3**	**1.3 ± 1.7**	**9.9 ± 3.4**

b) in-contact vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
B1	0.0 ± 0.0	0.6 ± 1.0	0.0 ± 0.0	11.7 ± 0.6
B2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12.0 ± 0.0
B3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	11.5 ± 1.0
B4	0.0 ± 0.0	0.1 ± 0.4	4.4 ± 4.2	7.9 ± 3.6
Total	**0.0 ± 0.0**	**0.3 ± 1.3**	**1.2 ± 2.2**	**9.7 ± 3.2**

c) Unvaccinated control chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.2 ± 2.5
C2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.3 ± 2.6
Total	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**8.2 ± 2.6**

*: Day of Challenge
SD: Standard deviation

Table 4.13. Mean ELISA optical density (OD) values for antibodies to NDV of pullets bled at 21, 35, 49 and 63 days of age

a) Eye-drop vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
A1	0.0 ± 0.0	0.0 ± 0 .0	0.1 ± 0.0	0.5 ± 0.1
A2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.3
A3	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.2
A4	0.0 ± 0.0	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.3
Total	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.1 ± 0.0**	**0.3 ± 0.2**

b) In-contact vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
B1	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2
B2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.0
B3	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.4 ± 0.1
B4	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.3
Total	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.1 ± 0.1**	**0.3 ± 0.2**

c) Unvaccinated control chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
C2	0.0 ± 0.0	0.0 ± 0.0	**0.0 ± 0.0**	0.1 ± 0.1
Total	**0.00± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.2 ± 0.1**

*: Day of Challenge
SD: Standard deviation

Table 4.14. Mean ELISA index values (IV) for antibodies to NDV of pullets bled at 21, 35, 49 and 63 days of age

a) Eye-drop vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
A1	0.2 ± 0.01	0.3 ± 0.03	0.3 ± 0.1	2.9 ± 0.9
A2	0.2 ± 0.09	0.2 ± 0.02	0.2 ± 0.1	3.5 ± 1.9
A3	0.2 ± 0.09	0.3 ± 0.04	0.2 ± 0.1	1.6 ± 1.1
A4	0.01 ±0.09	0.3 ± 0.02	0.4 ± 0.2	1.3 ± 2.1
Total	**0.2 ± 0.1**	**0.3 ± 0.02**	**0.3 ± 0.2**	**2.4 ± 1.8**

b) in-contact vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
B1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	2.4 ± 1.0
B2	0.2 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	3.5 ± 0.0
B3	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	2.2 ± 0.9
B4	0.2 ± 0.1	0.2 ± 0.0	0.5 ± 0.3	1.5 ± 1.6
Total	**0.2 ± 0.1**	**0.2 ± 0.0**	**0.3 ± 0.3**	**2.0 ± 1.4**

c) Unvaccinated control chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	1.4 ± 1.1
C2	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	1.2 ± 0.8
Total	**0.2 ± 0.1**	**0.2 ± 0.0**	**0.2 ± 0.1**	**1.3 ± 0.9**

*: Day of Challenge
SD: Standard deviation

Table 4.15. . Relative mean of ELISA antibody titres (Log_2) to NDV of pullets bleed at 21, 35, 49 and 63 days of age

a) Eye-drop vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
A1	3.4 ± 1.4	4.1 ± 0.0	4.1 ± 1.3	11.7 ± 1.5
A2	2.4 ± 1.5	3.9 ± 0.3	2.5 ± 1.5	12.1 ± 2.8
A3	2.5 ± 1.7	4.0 ± 0.5	3.6 ± 1.3	9.5 ± 2.8
A4	-1.0 ± 3.1	4.6 ± 1.5	4.8 ± 2.2	7.2 ± 3.7
Total	**1.8 ± 2.6**	**4.1 ± 0.8**	**3.7 ± 1.7**	**10.1 ± 3.3**

b) In-contact vaccinated chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
B1	3.5 ± 1.5	4.1 ± 0.5	2.8 ± 0.8	11.4 ± 1.2
B2	2.3 ± 1.4	4.0 ± 0.4	1.7 ± 0.6	12.8 ± 0.0
B3	2.2 ± 2.6	3.7 ± 0.1	2.4 ± 1.5	11.1 ± 1.6
B4	2.4 ± 1.3	3.8 ± 0.4	5.9 ± 2.0	8.1 ± 3.7
Total	**2.6 ± 1.7**	**3.9 ± 0.4**	**3.3 ± 2.1**	**9.8 ± 3.1**

c)Unvaccinated control chickens

	At day 21	At day 35	At day 49*	At day 63
Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C1	2.6 ± 1.4	3.9 ± 0.4	2.8 ± 1.6	0.4 ± 3.7
C2	2.1 ± 0.8	3.8 ± 0.8	2.9 ± 1.7	0.7 ± 2.6
Total	**2.4 ± 1.1**	**3.9 ± 0.4**	**2.8 ± 1.4**	**8.5 ± 3.2**

*: Day of Challenge
SD: Standard deviation

4.10.2. Seroconversion

The results of HI-NDV seroconversion are shown in Table 4.16.

Table 4.16. Results of HI-Seroconversion of pullets bled at 21, 35, 49 and 63 days of age

a) Eye-drop vaccinated chickens

	At day 21		At day 35		At day 49*		At day 63	
Group	No	Yes	No	Yes	No	Yes	No	Yes
A1	7	0	7	0	6	0	0	7
A2	7	0	7	0	6	0	0	7
A3	7	0	7	0	7	0	0	7
A4	7	0	6	1	3	4	0	7
Total	**28**	**0**	**27**	**1**	**24**	**4**	**0**	**28**

b) In-contact vaccinated chickens

	At day 21		At day 35		At day 49*		At day 63	
Group	No	Yes	No	Yes	No	Yes	No	Yes
B1	7	0	7	0	7	0	0	3
B2	7	0	7	0	7	0	0	1
B3	7	0	7	0	7	0	0	4
B4	8	0	8	0	2	6	0	8
Total	**29**	**0**	**29**	**0**	**23**	**6**	**0**	**16**

c) Unvaccinated control chickens

	At day 21		At day 35		At day 49*		At day 63	
Group	No	Yes	No	Yes	No	Yes	No	Yes
C1	16	0	16	0	16	0	0	11
C2	14	0	14	0	14	0	0	7
Total	**30**	**0**	**30**	**0**	**30**	**0**	**0**	**18**

*: Day of challenge

At day 49 of age, 14 % (4/28) of the chickens vaccinated by eye-drop and 20.7 (6/29) % of in- contact vaccinated chickens showed positive titres for HI-NDV antibody.

All surviving chickens at day 63 were seropositive for antibodies to HI-NDV NDV.

4.11. Protection induced by ND V4 vaccine against B1172 challenge NDV

The Table 4.17 shows the relationship between the HI-NDV antibody seroconversion at pre-challenge (at 43 days) and the ability to survive 14 days after challenge.

Of the surviving chickens, 4/28 (14 %) of chickens vaccinated by the eye-drop method and 6/16 (37.5 %) of those vaccinated by the in-contact method were seropositive for HI-NDV antibody on day 49. Among protected chickens, 24/28 (86 %) of chickens vaccinated by the eye-drop route, 10/16 (62.5 %) of chickens vaccinated by the in-contact method and 18/18 (100 %) in the unvaccinated control group were seronegative for HI-NDV antibody at pre-challenge day.

Table 4.17. Correlation between the HI-seroconversion at day 49 (at pre-challenge) and the protection induced in replacement pullets by ND V4 vaccine against B1172 NDV

a)Eye-drop vaccinated chickens

	Number of chickens						
		Dead			Protected		
Group	challenged	+	-	Total	+	-	Total
1	7	0	0	0	0	7	7
2	7	0	0	0	0	7	7
3	7	0	0	0	0	7	7
4	7	0	0	0	4	3	7
Total	**28**	**0**	**0**	**0**	**4**	**24**	**28**

b) In-contact vaccinated chickens

	Number of chickens						
		Dead			Protected		
Group	Challenged	+	-	Total	+	-	Total
1	7	0	4	4	0	3	3
2	7	0	6	6	0	1	1
3	7	0	3	3	0	4	4
4	8	0	0	0	6	2	8
Total	**29**	**0**	**13**	**13**	**6**	**10**	**16**

c) Unvaccinated control chickens

	Number of chickens						
		Dead			Protected		
Group	Challenged	+	-	Total	+	-	Total
1	16	0	5	5	0	11	11
2	14	0	7	7	0	7	7
Total	**30**	**0**	**12**	**12**	**0**	**18**	**18**

+: Positive to HI- NDV antibody seroconversion
-: Negative to HI- NDV antibody seroconversion

4.12. Correlation between HI test and ELISA test to detect antibodies for NDV

Values of sensitivity, specificity of the ELISA-NDV test and agreement beyond chance (K agreement) between the HI-NDV and the ELISA-NDV test are shown in Tables 4.18, 4.19, 4.20, 4.21 and 4.22.

Table 4.18. Comparison of the HI and ELISA tests for pullets bled at 21 days old

ELISA results	HI results		Total of Chickens
	Positive	Negative	
Positive	0	0	0
Negative	0	87	87
Total of chickens	0	87	87

Relative sensitivity and K coefficient cannot be determined. Relative specificity = 100%

Table 4.19. Comparison of the HI and ELISA tests for pullets bled at 35 days old

ELISA results	HI results		Total of Chickens
	Positive	Negative	
Positive	0	0	0
Negative	1	86	87
Total of chickens	1	86	87

Relative sensitivity and K coefficient cannot be statistically determined. Relative specificity = 100 %

Table 4.20. Comparison of the HI and ELISA tests for pullets bled at 49 days old

ELISA results	HI results		Total of Chickens
	Positive	Negative	
Positive	2	0	2
Negative	8	77	85
Total of chickens	10	77	87

Relative sensitivity = 20 %. Relative specificity = 100 %
K coefficient = 0.31

No agreement (K= 0.31) between the two tests was observed in serological results of blood samples taken at 49 days. An excellent agreement (K= 0.78) was observed with pooled data.

Table 4.21. Comparison of the HI and ELISA tests for pullets bled at 63 days old

ELISA results	HI results		Total of Chickens
	Positive	Negative	
Positive	49	0	49
Negative	13	0	13
Total of chickens	62	0	62

Relative specificity and K coefficient cannot be statistically determined.
Relative sensitivity = 79 %.

Table 4.22. Comparison of the HI and ELISA tests for pullets bled using a pooled data

ELISA results	HI results		Total of Chickens
	Positive	Negative	
Positive	51	0	51
Negative	22	250	272
Total of chickens	73	250	323

Relative sensitivity = 69.8 %.
Relative specificity = 100%
K coefficient = 0.78

4.13. Statistics

4.13.1. Testing for the mean temperature difference between isolator units throughout the experiment

The mean temperature difference between the isolator units is given in Tables 4.23, 4.24, 4.25 and 4.26.

The differences of mean temperature between isolators were statistically significant during the experiment ($p < 0.001$).

Table 4.23. Results of statistical analysis of the difference in mean temperature recorded for the period 1 to 7 days of age

Unit	t-test value	df	Sig.	Mean	Interval with 95 % of confidence	
					Lower	Upper
Unit 1	120.3	6	0.000	29.4	28.8	30
Unit 2.6	120.3	6	0.000	29.4	28.8	30
Unit 3	120.3	6	0.000	29.4	28.8	30
Unit4	120.3	6	0.000	29.4	28.8	30
Unit 7	120.3	6	0.000	29.4	28.8	30
Unit 8	120.3	6	0.000	29.4	28.8	30

t: student's t-test value.
df: degree of freedom
Sig.: Statistical significance

Table 4.24. Results of statistical analysis of the difference in mean temperature recorded for the period 8 to 14 days of age

Unit	t-test Value	df	Sig.	Mean	Interval with 95 % of confidence	
					Lower	Upper
Unit 1	82.4	6	0.000	26.8	26	27.6
Unit 2.6	82.4	6	0.000	26.8	26	27.6
Unit 3	82.4	6	0.000	26.8	26	27.6
Unit4	82.4	6	0.000	26.8	26	27.6
Unit 7	7.6	6	0.000	30.8	21	40.7
Unit 8	82.4	6	0.000	26.8	26	27.6

t: Student's t-test value.
df: degree of freedom
Sig.: Statistical significance

Table 4.25. Results of statistical analysis of the difference in mean temperature recorded for the period 15 to 21 days of age

Unit	t-test Value	df	Sign.	Mean	Interval with 95 % of confidence Lower	Upper
Unit 1	100.2	6	0.000	24.1	23.5	24.7
Unit 2	131.6	6	0.000	24.4	24.9	24.8
Unit 3	128.1	6	0.000	24.3	23.8	24.7
Unit 4	128.1	6	0.000	24.3	23.8	24.7
Unit 7	128.1	6	0.000	24.3	23.8	24.7
Unit 8	128.1	6	0.000	24.3	23.8	24.7

t: Student's t-test value.
df: degree of freedom
Sig.: Statistical significance

Table 4.26. Results of statistical analysis of the difference in mean temperature recorded for the period 22 to 63 days of age

Unit	t-test value	df	Sign.	Mean	Interval with 95 % confidence Lower	Upper
Unit 1	226.8	41	0.000	21.4	21.3	21.6
Unit 2.6	207.4	41	0.000	21.5	21.3	21.8
Unit 3	175.3	41	0.000	22.0	21.8	22.3
Unit4	239.7	41	0.000	21.6	21.4	21.8
Unit 7	253.2	41	0.000	22.0	21.8	22.2
Unit 8	220.3	41	0.000	22.3	22.1	22.5

t: Student's t-test value.
df: degree of freedom
Sig.: Statistical significance

4.13.2. Statistical analysis of percentage survival after challenge

The statistical analysis of percentage survival after challenge is given in Table 4.27.

The statistical analysis indicates that the difference in percentage of survivals was significant between the groups of chickens vaccinated by eye-drop route and the groups of chickens vaccinated by the in-contact method ($p < 0.001$) and between unvaccinated control chickens and chickens vaccinated by the eye-drop route ($p < 0.0001$). The results of challenge were not significant ($P > 0.5$) between the unvaccinated control group and group of chickens vaccinated by in-contact method.

Table 4.27. Results of the Chi-square and Fisher Exact tests for the results of challenge

Groups	X^2	df	X^2 p-value	Fisher Exact test p- value
Unvaccinated control/ Eye-drop vaccinated	11.8	1	0.0006	0.0001
Unvaccinated control/ in-contact	3.0	1	0.08	0.8
Eye-drop vaccinated/ in-contact group	13.8	1	0.0002	0.001

x^2: Chi-square
df: Degree of freedom

4.13.3. Testing for the differences in the appearance of clinical signs and gross lesions in dead or sacrificed chickens

The results of statistical analysis for the difference in appearance of clinical signs and gross lesions after challenge with B1172 NDV are shown in Tables 4.28 and 4.29.

Table 4.28. Results of testing the difference in appearance of clinical signs and gross lesions between chickens that died following challenge with the B1172 NDV

	Clinical signs			Respiratory tract lesions		
Groups	X^2	X^2 P- value	F. Exact P-value	X^2	X^2P-value	F. Exact p - value
Control/ Eye-drop vaccinated	a)	a)	a)	a)	a)	a)
Control/ in-contact	a)	a)	a)	0.5	0.5	1.0
Eye-drop vaccinated control	a)	a)	a)	a)	a)	a)

	Gastrointestinal tract lesions		
Statistical Group	X^2	X^2 P- value	F. Exact P-value
Control/ Eye-drop vaccinated	a)	a)	a)
Control/ in-contact	2.3	0.15	0.09
Eye-drop vaccinated/ in-contact	a)	a)	a)

x^2: Chi-square. F. Exact: Fisher exact tests (Two tailed P- value)
a): Not valid because one of the row and column has value > 5.

Table 4.29. Results of testing the difference in appearance of clinical signs and gross lesions between chickens that survived challenge with the B1172 NDV

	Clinical signs			Respiratory tract lesions	
Groups	X^2	X^2 P- value	F. Exact P-value	X^2	X^2P-value
Control/ Eye-drop vaccinated	1.7	a)	0.1	3.1	0.07
Control/ in-contact	1.4	a)	1.0	5.7	0.02
Eye-drop vaccinated/ in-contact	1.3	a)	0.2	0.5	0.5

	Gastrointestinal tract lesions	
Groups	X^2	X^2 P- value
Control/ Eye-drop vaccinated	1.5	0.2
Control/ in-contact	1.8	0.2
Eye-drop vaccinated/ in-contact	0	1.0

x^2: Chi-square.
F. Exact: Fisher exact test (Two tailed P- value)
a): Not valid because X^2 has value <5.

In dead chickens, the difference in appearance of clinical and gross lesions was not statistically significant ($P > 0.05$) for all groups compared. In surviving chickens, the difference was only significant in the appearance of respiratory tract lesions ($P < 0.05$) between the unvaccinated control group and group of chickens vaccinated by in-contact method.

4.13.4. Testing for the difference in NDV isolation between chickens that died and survived after challenge with B1172 NDV

The statistical analysis for the differences in the NDV isolation between chickens that died and survived after challenge with B1172 NDV is indicated in Tables 4.30 and 4.31.

The results of NDV isolation were not significant between any groups of chickens that died after challenge. However, in surviving chickens, the results were significant ($P < 0.001$) between unvaccinated control group and the eye-drop vaccinated group and significant ($p < 0.01$) between unvaccinated control groups and chickens vaccinated by the in-contact method.

4.13.5. Testing for the difference in the bacterial isolation from samples collected at necropsy

The bacterial isolation in samples collected from each bird at necropsy was not significantly different between groups (Table 4.32).

Table 4.30. Statistical analysis for the differences in NDV isolation between chickens that died after challenge with B1172 NDV

Groups	X^2	df	X^2 p-value
Control/ Eye-drop vaccinated	a)	1	a)
Control/ in-contact	0.4	1	0.6
Eye-drop vaccinated/ in-contact group	a)	1	a)

X^2: Chi-square.
df: Degree of freedom
a): Not valid because X^2 has value <5

4.13.6. Testing for the difference in the HI-NDV antibody mean titer between groups

Tables 4.33, 4.34 and 4.35 show the results of the Student's t test for the differences in HI-NDV antibody titer between groups.

At 35 days of age, the difference in the HI-NDV means titres was not significant between any groups compared.

Table 4.31. Statistical analysis for the differences in the NDV isolation between chickens that survived challenge with B1172 NDV

Groups	X^2	df	X^2 p-value	Fisher Exact test p-value[1]
Control/ Eye-drop vaccinated	12.3	1	0.001	0.0001
Control/ in-contact	7.0	1	0.9	0.003
Eye-drop vaccinated/ in-contact group	a)	1	a)	a)

X^2: Chi-square
df: Degree of freedom
a): Not valid because X^2 has value <5
1: Two tailed p- value

Table 4.32. Results of statistical analysis of bacterial isolation following challenge with B1172 NDV

Groups	X^2	df	X^2 P-value
Control/ Eye-drop vaccinated	a)	1	0.8
Control/ in-contact	0.15	1	0.7
Eye-drop vaccinated/ in-contact group	0.07	1	0.8

X^2: Chi-square
df: Degree of freedom
a): Not valid because X^2 has value <5

Table 4.33. Results of statistical analysis of mean difference in HI-NDV antibody titer between groups at 35 of age

Groups	N	Mean	Std[1]	t-test value	df	Sig.
Control/ Eye-drop vaccinated	30 28	0.00 0.36	0.00 1.34	-1.5	56	0.15
Control/ in-contact	30 29	0.00 0.17	0.00 0.54	-1.7	56	0.08
Eye-drop vaccinated/ in-contact group	28 29	0.36 0.17	1.34 0.54	-0.7	35	0.5

N: Number of chickens.
t: Student t-test.
Sig.: Statistical Significance
Std: Standard deviation mean

Table 4.34. Results of statistical analysis of mean difference in HI-NDV antibody titer between groups at day 49 of age

Groups	N	Mean	Std	t-test value	df	Sig.
Control/ Eye-drop vaccinated	30 28	0.00 2.8	0.00 2.1	-3.1	56	0
Control/ in-contact	30 29	0.00 1.21	0.00 2.18	3.04	57	0
Eye-drop vaccinated/ in-contact group	28 29	2.8 1.21	2.1 2.18	-0.7	49	0.47

N: Number of chickens.
t: Student's t-test
Sig.: Statistical significance.
Std: Standard deviation

At 49 days of age, the difference of HI-NDV antibody mean titer was highly significant ($P < 0.001$) between the unvaccinated control chickens and chickens vaccinated by the eye-drop method as well as between the unvaccinated control groups and chickens vaccinated by the in-contact method. No significant difference was found between groups of chickens vaccinated by the eye-drop method and chickens vaccinated by the in-contact method ($p > 0.5$).

Table 4.35. Results of statistical analysis of mean difference in HI-NDV antibody titer between groups at day 63 of age

Groups	N	Mean	Std	t-test value	df	Sig.
Unvaccinated control/ Eye-drop vaccinated	18 28	8.61 9.86	2.57 3.36	1.42	42	0.16
Unvaccinated control/ in-contact	18 16	8.61 9.75	2.57 3.15	1.14	29	0.26
Eye-drop vaccinated/ in-contact group	18 16	8.61 9.75	2.57 3.15	-0.1	33	0.91

N: Number of chickens.
t: Student's t-test
Sig.: statistical significance
Std: Standard deviation mean

At day 63, no significant difference in HI-NDV antibody titer was seen between groups.

4.13.7. Statistical analysis for the difference in the protection between the groups induced by ND V4 vaccine against B1172 challenge NDV

Statistical analysis for the difference in the protection induced by ND V4 vaccine against B1172 challenge NDV between groups is given in Table 4.36.

Table 4.36. Statistical analysis for the difference in the protection induced by ND V4 vaccine against B1172 challenge NDV between groups

Groups	X^2	df	X^2 p-value
Control/ Eye-drop vaccinated	17.6	1	0.001
Control/ in-contact	12.6	1	0.01
Eye-drop vaccinated/ in-contact group	20	1	0.0001

X^2: Chi-square
df: Degree of freedom

The level of protection between chickens vaccinated by the eye-drop route and chickens vaccinated by the in-contact method was significant at level of $P < 0.0001$ and between unvaccinated control group chickens and chickens vaccinated by the eye-drop route the significance was at level of $P < 0.001$. The results of protection between the unvaccinated control group and chickens vaccinated by the eye-drop route were also significant ($P < 0.01$).

CHAPTER 5: DISCUSSION

The manufacturing company (Arthur Webster, PTY) recommends using ND V4 vaccine possessing 10^6 EID_{50} of infectivity in order to provide good protection against velogenic NDV. In this experiment, the mean titer of the ND V4 vaccine was $10^{7.2}$ EID_{50} (Table 4.4). This titer was within the requirements recommended by the manufacturing company and by other researchers (Spradbrow 1987, Heath e*t al*. 1992).

The V4 strain of NDV used in this experiment did not kill any inoculated chicken embryo. These results confirm the apathogenicity of the ND V4 strain, initially reported by Simons (1967) and supported later by several authors, including Spradbrow & Samuel (1991), Heath *et al.* (1992).

Several studies (Heath *et al.* 1992), with ND V4 vaccine have demonstrated the safety of the ND V4 vaccine in the vaccinated chickens. Similar findings were observed in this experiment. Chickens remained healthy after vaccination with ND V4 vaccine. These results confirm the inclusion of the ND V4 strain within the asymptomatic or apathogenic pathotype as described by Beard and Hanson (1984, cited by Alexander 1991).

The HI-NDV antibody titer was not detected at day 21 and occasionally at 35 days of age. A slight rise of HI-NDV antibody was observed in some birds at 49 days of age. French (1969) had observed that a weak NDV antibody immune

response occurring after primary vaccination with ND V4 vaccine was boosted after secondary vaccination. Thereafter, similar results were reported by others authors including Ibrahim *et al.* 1981, Bell (1991) and Samuel & Spradbrow (1991). No significant difference was found (P>0.5) between the eye-vaccinated group and the in-contact group (Tables 43, 34). The explanation for this may be lack of dose dependence. Vaccinated birds within a closed environment excreted the ND V4 virus and all birds (both vaccinated and in contact) were re- infected simultaneously, due to high transmissibility of the ND V4V strain between vaccinated chickens and unvaccinated chickens kept in contact.

Chickens that displayed a moderate titer at pre-challenge displayed less HI-NDV titer after challenge (Spalatin *et al.* 1976 and Spradbrow *et al*. 1978, 1980). Similar findings were observed in this experiment (Tables 4.12 and 35). However, the results were not statistically significant in any groups (Table 4.35). Probably challenge dose (Alexander 1997) or individual variation in the immune response (Turner *et al.* 1976, Jayawardane *et al.* 1990, Alexander 1991, Bell *et al.* 1991) rather than hummoral immune system respose have played a role. A similar explanation may be given for the lower HI- antibody titer seen in the unvaccinated control C1 group (Table4.12) compared to C2. However, the protection acquired by C1 was higher than C2.

All surviving birds showed positive titres to HI-NDV antibody (Table 4.35). Similar results were reported in others experiments (Turner *et al.* 1976, 1977).

This is an indication of survival of vaccinated chickens from an infection of highly velogenic NDV (Allan, Lancaster & Toth 1978, cited by 1978 Alexander 1997). No significant difference was seen between groups (Table 4.35).

During this experiment, 100 % of embryo mortality was observed 48 to 72 hours after egg inoculation with the challenge B1172 NDV. After challenge, dyspnoea and mucoid fluid discharge were evident in the pullets that died or remained sick until that they were sacrificed (Table 4.8 and 4.9). Tests carried out at the ND-Reference Laboratory (Central Veterinary Laboratory-Weybridge), to characterize B1172 NDV and the re-isolated NDV gave an IVPI of 2.17 (Manvell, 1993 personal communication).

The MDT of the freeze-dried B1172 NDV and the clinical signs seen after challenge B11722 NDV indicate that the challenge virus is a velogenic pathotype of NDV described by Beard and Hanson (1984, cited by Alexander 1991, Alexander 1995b).

The gastrointestinal lesions observed during this trial were not reported during the outbreak of 1993, although they are not specific lesions caused by the NDV infection (Alexander 1991).

Results of challenge with velogenic ND virus shown in Table 4.7 demonstrate that birds vaccinated with V4 vaccine acquired protection to challenge against B1172 NDV. These results confirm that V4 vaccine induce protection against a

wide range of velogenic NDV (Heath *et al.* 1992, Spradbrow 1993/4) including the B1172 NDV isolate.

As B1172 NDV is innately velogenic, severe clinical signs and gross lesions with a high mortality were expected to appear after challenge at the least in the unvaccinated control group. However, only 14/30 unvaccinated control chickens showed clinical signs after challenge. Of these, 12/30 died. Similar results were reported by Jayawardane *et al.* (1990). It is possible that challenge dose-dependence, or accidental delivery of the challenge virus into the upper oesophageal region in instead of to the anterior tracheal region occurred during the challenge procedures (Marquardt *et al.* 1985, Alexander 1991). However, mortality from 14% to 100% occurred within groups of chickens vaccinated by in-contact method. The differences in the % of survivals (Tables 4.7 and 4.22) between the unvaccinated control group and in-contact group were not significant (P. 0.5). It is also possible that an accidental transmission of NDV V4 strain to the control group occurred through attendants' clothing or an indirect exposure due to environmental contamination. The following pre-disposing factors may have played a role for that contamination:

a) The isolator units for the vaccinated groups as well as for the unvaccinated control chickens were kept closed within the same experimental unit and both served by the same attendant;

b) Ration used for feeding vaccinated and unvaccinated control chickens was taken from the same bags that were stored within the experimental shed;

c) Although measures for biosecurity described elsewhere were taken, contaminated litter from isolator units holding vaccinated groups was cleaned out using open devices;

d) During the vaccinations, all groups/chickens were removed out from their isolator units because of the troubles experienced in handling the pullets while they were inside of the isolator units;

e) The results of clinical signs, respiratory lesions and the level protection were not statically significant different ($p > 0.5$) between the unvaccinated control group and the group of chickens vaccinated by the in-contact method (Tables 4.7, 4.8, 4.9, 4.10,4.11, 4.22, 4.27, 4.29, and 4.30).

The transmissibility of the NDV V4 strain between vaccinated chickens in close contact with the unvaccinated chickens has been published several times. Turner *et al.* (1976) observed that seven chickens vaccinated by V4 vaccine and kept in direct contact with 151 adult chickens resisted 100 % protection of the whole batch. Other authors including Heath et al. (1992) and Alders et al. (1994) reported similar results. The present study showed that groups of unvaccinated chickens that were in direct contact with those vaccinated with ND V4 vaccine were positive to HI-NDV test (Table 4.12) and the protection level acquired was between 14 % - 100% respectively.

Tests to identify the presence of NDV V4 strain there not attempted in this experiment. However, the results of MDT and HA titres from NDV isolations of birds died after challenge associated to the clinical signs similar those observed during the field outbreak indicate that the velogenic ND virus isolated ND was the challenge B1172 NDV.

Data from the re-isolation of NDV indicate that no re-isolation of the NDV resulted from chickens vaccinated by the eye-drop method. In surviving chickens, NDV was recovered only from the unvaccinated control group. The results were only significant between groups that survived challenge ($P<0.01$).

No harmful bacterial infection was detected from tissues sampled from individual birds at necropsy. The results were not statistically significant between groups. The significance of the present findings demonstrates that no bacterial infectious diseases occurred during this experiment.

Lack of relationship between the protection degree and the titer of HI-NDV antibodies induced by ND V4 vaccine has been published in several studies. Turner, *et al.* (1976) showed that either chickens possessing no detectable HI-NDV antibody or chickens with HI-NDV antibody titer of $Log_2 \leq 2$ or $Log_2 > 2$ at pre-challenge were able to resist challenge. These results are in general agreement with those were reported by Spradbrow *et al.* (1990), Bell *et al.* (1991b), Heath *et al.* (1992), and Jayawardane & Samuel (1995a). Similar results were also observed in this study (Table 4.22 and 4.37). At pre-challenge (on 49 days of age), 85.7 % of chickens vaccinated by the eye-drop

route had no detectable HI-NDV antibody titer 14 days after they survived challenge. In the group of chickens vaccinated by the in-contact method, 62.5 % of birds did not display any HI antibody at pre-challenge but they also survived challenge. In the unvaccinated control groups, all chickens were negative to HI-NDV test at pre-challenge but they were protected against the B1172 NDV. Local immunity and cell- mediated immunity (Timms & Alexander cited by Heath *et al.* 1992 Jayawardane & Spradbrow 1995a,b) may play a role as a part of immune response in protection induced by ND V4 vaccine.

Evaluation of local immunity and cell-mediated immunity were outside the scope of this experiment.

The V4 ND vaccine can be administered by all conventional routes (Eye-drop method, intranasal, intramuscular, drinking water) and the high efficacy of the eye-drop has been reported (Heath *et al*. 1992) and Bell *et al.* (1991ab). In the present study there was also evidence that vaccination by the eye-drop route was more efficacious than in-contact administration.

Tables 4.18 4.19, 4.20, 4.21 and 4.22 demonstrate that the sensitivity of ELISA-NDV against the HI-NDV test was ranged between 0.21 % - 0.80 % and 100 % specificity than the HI-NDV. The agreement (Kappa) beyond chance between two tests was K= 0.31 at day 49 at pre-challenge day, K= 0.80 at 63 days of age and K= 0.78 when pooled data were used. As Kappa values of “0” indicate no agreement and “1” indicate a perfect agreement, this means that there was a fair agreement between the two tests on serum samples taken at

day 49 and a good agreement between two tests on serum samples taken from surviving chickens and when pooled data were used.

Few studies using the Kappa agreement test to compare the HI-NDV and ELISA-NDV have been reported. The present study demonstrated that there is moderate to a good agreement between the two tests. (Marquardt *et al*. (1985), Brown *et al*. (1990) and Cvelić-Čabrilo *et al*. (1992), postulated that experimental conditions (small-sized samples, sample timing post-infection, dose of vaccine or route of vaccination, difference in positive-negative cut-off values may affect the results of the comparison between ELISA-NDV and HI-NDV tests. The use of La Sota NDV as antigen in performing HI-NDV test may result in an overestimation of protective serum antibody (Maas *et al.* 1998). It is possible that the lower titer of HI-antibodies induced by ND V4 vaccine, the small sample size and the use of *La Sota* antigen in performing HI-NDV test ($Log_2 \geq 4$) may have affected the sensitivity values observed in this comparison.

CHAPTER 6: CONCLUSIONS

Newcastle disease is endemic in most developing countries in Asia and Africa where control of the disease is hampered by lack of affordable thermostable vaccines. ND V4 and its derivative ND V4HR were developed to assist in the control of ND in such areas (Ideris *et al.* 1990; Jayawardane *et al.* 1990; Bell *et al.* 1991a,b; Spradbrow 1993/4; Bell *et al.* 1995). ND V4 vaccine was originally developed for use in the Australian poultry industry, but together with its derivative ND V4HR vaccine, has been used to assist in the control of ND in many countries. These vaccines possess good immunogenicity and transmissibility and are easily administered by all conventional routes as well as mixed in food. Moreover, ND V4 and ND V4HR are thermostable; allowing their use in rural areas and especially in tropical regions where a suitable cold chain required for proper conservation of conventional vaccines is lacking. The protection afforded by ND V4 and ND V4HR against challenge in the field and in the laboratory has been demonstrated in several countries (French *et al.* 1969, Turner *et al.* 1976, Spradbrow *et al.* 1978, Spradbrow *et al.* 1980, Sagild & Spalatin 1982;Westbury *et al.* 1984; Ibrahim *et al.* 1987; Jayawardane *et al.* 1990; Bell *et al.* 1991; Jagne *et al.* 1991; Samuel & Spradbrow 1991; Alders *et al.* 1994; Sagild & Haresnape 1987; Bell *et al.* 1995).

A pneumotropic velogenic strain of NDV designated B1172/93, was isolated from a severe outbreak of ND that occurred on South African farms in 1993. This outbreak was characterised by a severe haemorrhagic tracheitis with mortality up to 90 % (Coetzee personal communication, 1993).

One of the objectives of the studies reported in this thesis was to evaluate the safety and the efficacy of ND V4 vaccine against challenge with ND B1172 virus isolate

These studies confirm that the ND V4 vaccine strain is:

(a) Apathogenic, producing no clinical signs or gross lesions in vaccinated birds,

(b) Immunogenic, producing a humoral immune response, which could be detected by the HI test. Vaccinated birds with low or undetectable HI-NDV titer at pre-challenge survived challenge with isolate B1172. This suggests that ND V4 stimulates local immunity and cell mediated immunity as well as humoral immunity, confirming the findings of others (Turner *et al.* 1976; Spradbrow *al.* 1990; Bell *et al.* 1991b; Timms & Alexander cited by Heath *et al.* 1992; Jayawardane & Spradbrow 1995a,b),

(c) Transmissible, spreading from vaccinated to in-contact chickens and, probably inadvertently to control chickens. Although in-contact chickens did develop antibody, vaccination by eye drop was shown to be a more reliable

method of protecting chickens from challenge with virulent NDV and

(d) Efficacious, providing protection against the pneumotropic B1172 ND virus.

The B1172 isolate was shown to behave in a similar manner to the field virus isolated during the outbreak of Newcastle disease that occurred in South Africa in 1973. Clinical signs and gross lesions observed in challenged birds that succumbed to infection confirmed the pneumotropism of the isolate. Virus was also isolated from tissues of infected birds.

We also found that not all unvaccinated control chickens always died when challenged with B1172. A number of factors may have been responsible for this, including insufficient dose of challenge virus, inappropriate route of challenge or, more likely, spread of the ND V4 vaccine strain to unvaccinated control chickens through inadequate biosecurity procedures (Marquardt *et al.* 1985; Alexander 1991).

Serological tests for antibodies to NDV may be used to detect infection with virus or to monitor vaccinations. Most techniques used for the detection of antibody to virus have been applied to NDV (Tizzard 1982; Alexander 1991). Conventionally the HI test is the most commonly used serological test to assess the efficacy of vaccination and to assess the level of protection afforded by vaccination (Alexander 1991). Recently, a semi-automated ELISA

has been developed to detect antibodies to NDV and studies have been undertaken to compare results obtained using these two methods (Miers *et al*. 1983; Snyder *et al*. 1983; Marquardt *et al*. 1985; Thayer *et al*. 1987a,b; Adair *et al*. 1989; Brown *et al*. 1990; De Witt *et al*. 1992; Cvelić *et al*. 1992; Czifra *et al*. 1996, 1998).

The second objective of the studies reported in this dissertation was to compare the HI and ELISA tests for antibodies to NDV.
Results of the comparison between the HI and ELISA tests showed that:

1. The ELISA-NDV test appears to be more sensitive and specific relative to the HI-NDV test,

2. There is a fair to moderate agreement between the values obtained from the HI-NDV and ELISA-NDV, and

3. The HI-NDV test is easier to perform than the ELISA-NDV, which requires sophisticated equipment and trained personnel.

Some factors may have influenced the results of this comparison. These include the poor reproducibility of the HI test (Alexander 1991; Czifra *et al.* 1998) and the differences of experimental conditions (Marquardt *et al* 1985; Adair *et al* 1989; Brown *et al.* 1990; Cvelić *et al.* 1992; Maas *et al.* 1998).

CHAPTER 7: RECCOMENDATIONS

Short-cycle species such as poultry, goats, sheep and pigs might constitute a main source of income generation and add stability to overall farming systems, especially in developing countries.

Keeping poultry in developing countries and even in some developed countries has often been hampered by to ND. Newcastle disease is endemic in Asian and African countries.

Massive vaccination using conventional vaccines combined with strict biosecurity and good hygiene are feasible measures used in commercial poultry farms for the control and prevention of ND. However, thermostable ND V4 and ND V4-HR vaccines have been used in vaccination of household chickens.

The protection afforded by ND V4 and ND V4HR to challenge with a wide range of velogenic NDV has been recognised in several countries (French *et al*. 1969; Turner *et al*. 1976; Spradbrow *et al*. 1978; Spradbrow *et al*. 1980; Sagild & Spalatin 1982; Westbury *et al*. 1984; Ibrahim *et al.* 1987; Jayawardane *et al.* 1990; Bell *et al*. 1991a,b; Jagne *et al*. 1991; Samuel & Spradbrow 1991; Alders *et al*. 1994; Sagild & Haresnape 1987; Bell *et al*. 1995).

The safety, immunogenicity, transmissibility and thermostability of the ND V4 strain were also demonstrated in this study. Thus, the following recommendations are made:

a) Efforts to the use of V4 and V4HR vaccines in rural areas will be advantageous,

b) The eye drop method is the method of choice when individual vaccination is to be undertaken and

c) Strict measures for biosecurity must be considered in designing further experiments for the evaluation of ND V4 or ND V4HR vaccines.

The disease patterns of the B1172/93 NDV were demonstrated to be similar to the field virus isolated during the outbreak of ND that occurred in South Africa in 1973. However, further studies to re- evaluate the properties of the B1172 NDV are needed and these might include titration for the infectivity of 50 % of chicken lethal dose (CLD_{50}) by intracheal inoculation.

The feasibility, the specificity and sensitivity of the ELISA in relation to HI test in the detection of antibodies to NDV were also confirmed in the present study. However, some of questions that arose from the comparison of the HI test and ELISA test need further studies for explanation. Therefore, the following recommendations can be made:

a) The ELISA test might be employed at main Central Veterinary laboratories where many serological tests are demanded and stationer services available. In contrast, the HI test is advised for use at regional laboratories and where financial support are lacking and trained staff are usually not available,

b) It is relevant to standardise the HI-NDV test using reagents that comply with international standards (Alexander 1991; Maas *et al.* 1998) and

c) Further studies are required to explain the influence of the sample size, sampling time dependence, vaccine dose dependence, route of vaccine administration and antigenic difference of NDV on the comparison between the NDV-HI test and NDV- ELISA test (Adair *et al* 1989, Brown *et al.* 1990, Alexander 1991, Cvelić *et al.* 1992, Czifra *et al.* 1996, 1998).

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