AN EPIDEMIOLOGICAL SURVEY OF NEWCASTLE DISEASE VIRUS IN SOUTH AFRICA

by

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B.Sc. Hons. (U.F.S.)

Submitted in accordance with the academic requirements for the degree

MAGISTER SCIENTIAE

May 2001

In the Department of Microbiology and Biochemistry
Faculty of Agricultural and Natural Sciences
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DECLARATION

I, Barbara Keitumetse Mashope hereby declare that the dissertation hereby submitted by me for the Magister Scientiae degree at the University of the Orange Free State is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore cede copyright of the dissertation in favor of the University of the Free State. Appropriate acknowledgements in the text have been made where use of work, conducted by others, has been included.

The experimental work conducted and discussed in this thesis was carried out in the Department of Microbiology and Biochemistry, University of the Free State, Bloemfontein. The study was conducted during the period January 1999 to May 2001 under the supervision of Dr. J. Albertyn, and co-supervision of Drs. R.R. Bragg and E.van Heerden, of the Department of Microbiology and Biochemistry.

Signature: _____

Barbara K. Mashope

Date: May 2001

This work is dedicated to my parents Mr. and Mrs. G.M. Mashope, my siblings Boitumelo Bridgitte Mashope, Robert Thato Mashope, and the Sehurutshi family, especially my grandmother Mrs. D.L. Sehurutshi.

ACKNOWLEDGEMENTS

I would like to express and convey my sincere gratitude to all who assisted and contributed to the successful completion of this study. Included are the following:

Most importantly: "I thank the Great Spirit of the Universe for regularly carrying me to the edge, permitting me to contemplate the drop, and for holding me well. Ho!" (A. Walker),

Dr. J. Albertyn for his continued guidance, support, never ending patience and encouragement during the course of the study;

Drs. R.R. Bragg and E. Van Heerden for their useful insight and expertise;

Mrs. S. Cooper and the E.M. staff (Darlene & Valerie), Electron Microscopy Unit, Anatomical Pathology Division, Universitas Hospital, Bleomfontein, for their tireless support, technical assistance and unequalled expertise,

Mr. M. Cooper, (Virology Unit, Universitas Hospital, Bloemfontein), for his advise on the preparation of the viral isolates,

Drs. M. Wolfhaardt, Vergeer, and especially Ms. K. Klein (National Control Laboratory, Bloemfontein) for her enthusiasm, encouragement and assistance,

The academic and non-academic staff, and the students of the Department of Microbiology and Biochemistry, (UFS) for creating an atmosphere inducive for research and personal growth,

My wonderful parents, loving family and supportive friends, for their unconditional support, and the NRF for funding my studies

LIST OF ABBREVIATIONS

Ab	Antibody
A-PMV	Avian Paramyxovirus
Arg	Arginine
Asn	Asparagine
Av	Avian
CHO	Carbohydrate
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immusorbent Assay
EM	Electron microscopy
F	NDV Fusion glycoprotein
Gly	Glycine
HA	Haemagglutinin/ Haemagglutination
HI	Haemagglutination inhibition
HN	NDV Haemagglutinin neuraminidase glycoprotein
ICPI	Intracerebral Pathogenicity Index Test
ICTV	International Committee for Viral Taxonomy
IPT	Immunoperoxidase Test
IVPI	Intravenous Pathogenicity Index Test
Lys	Lysine
M	NVD Matrix protein gene
mAb	Monoclonal antibody
MDT	Mean Death Time
mRNA	Messenger RNA
NA	Neuraminidase
ND	Newcastle disease
NDV	Newcastle disease virus
NDV-AV	NDV-Australia Victoria
NLS	Nuclear localisation signal
nt	nucleotide

OP	Ondertsepoort Veterinary Institute
Ostr	Ostrich
PCR	Polymerase Chain Reaction
PMV	Paramyxovirus
RBC	Red Blood Cell
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription Polymerase Chain Reaction
SA	South Africa
Ser	Serine
SV	Sendai virus
Thr	Threonine
UP	University of Pretoria

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CHAPTER 1

INTRODUCTION

Newcastle disease (ND) is one of the most important diseases in poultry worldwide (King & Seal, 1997). Its importance is mainly due to the resulting economic losses that occur upon infection with virulent strains (Vewoerd, 1997). In South Africa, for example an epizootic that occurred in 1993/1998 caused one million broiler mortalities per week during its peak in 1994 (Coetzee, 1994) as cited by Verwoerd, (1997). This has prompted its inclusion in the Animal disease Control Acts (In South Africa: Act 35 of 1984) of most countries (Verwoerd, 1997).

This disease was first described in 1927 near Newcastle-On Tyne in England, hence the name (Gordon & Tindall, 1997). The ensuing worldwide trade in poultry made possible by the advent of refrigerated transport enabled the virus to spread to nearly every part of the civilized world (Gordon & Tindall, 1997).

Three main ND panzootics have occurred, each of which was caused by different strains. After the second panzootic in the 1960s' more emphasis was placed on vaccine development, and stricter control measures implemented (Jordan, 1990). The implementation of these control measures in the majority of the first world countries, resulted in their designation as NDV free regions. The majority of third world countries are currently classified as being endemic for NDV. These include South Africa, Asia, and Central and South America (Copland, 1987; Spradbrow, 1988; Rweyemamu *et al.*, 1991).

ND has a wide host range and is highly transmissible, necessitating the implementation of control measures to protect poultry from infection with virulent strains (Alexander *et al*, 1985). These measures include stamping out, zoning and vaccination (Verwoerd, 1997). In South Africa however, ongoing epizootics occur frequently in vaccinated commercial poultry (Herczeg *et al.*, 1999), highlighting the need for the development of effective control measures in this region (Verwoerd, 1997).

It has been inferred from serological surveys, and virus isolation investigations that in South Africa rural poultry populations, wild/migrating birds, and exotic birds harbour velogenic NDV strains (Verwoerd, 1997). These birds act as a constant source of infection for commercial poultry in close proximity, and via transmission by human contact and negligence (Verwoerd, 1997). It is feared, that vaccination may not confer sufficient protection to commercial poultry infected with unknown velogenic strains or old strains that have mutated (Calnek, 1991).

Rapid and unambiguous identification of aetiological agents can increase the effectiveness of control measures (Verwoerd, 1997). Currently, conventional diagnostic tests require virus isolation and the determination of the mean death time in eggs (MDT), the intracerebral pathogenicity index (ICPI) in one-day-old chicks, or the intravenous pathogenicity index (IVPI) in six week-old chickens, for accurate diagnosis (Alexander, 1991; Vewoerd, 1997). These techniques require up to four weeks to reach a positive diagnosis (Cavanagh, 1985), allowing the disease to spread with increasing mortality.

Subsequently, alternative strain differentiation techniques based on viral biological properties such as pathogenicity tests based on plaque formation, and physicochemical tests were developed (Cavanagh, 1985). The application of monoclonal antibody technology in strain differentiation enabled the establishment of epizootiologically meaningful groups (Alexander *et al.*, 1997).

Subsequently comparisons of nucleotide sequences of the HN and F genes of 11 NDV strains identified three distinct evolutionary lineages (Toyoda *et al.* 1987). Further research, led to the application of RT-PCR detection, which allowed the grouping of more than two hundred NDV strains into six epizootiologically meaningful groups (Ballagi-Pordány *et al.*, 1996). Unique fingerprints of vaccine strains are also possible using this method (Ballagi-Pordány *et al.*, 1996).

Two novel genetic groups VIIb and VIII, were revealed when this technique was employed to screen NDV isolates collected during epizootics in SA and Mozambique between 1990-1995 (Herczeg *et al.*, 1999). It is thought that genotype VIII viruses where maintained in the country

by endemic infection, whilst those belonging to VIIb reached Southern Europe (Herczeg *et al.*, 1999).

The purpose of this study was to use the above-mentioned RT-PCR based detection method to screen isolates implicated in an ND outbreak in commercial poultry in South Africa in 1998. Isolates were collected from various sectors of the poultry industry, and their genotypes determined. The results obtained from this study will contribute towards a worldwide epidemiological survey of ND being done in collaboration with researchers in Hungary.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Newcastle disease, one of the most infectious viral diseases in poultry (Alexander, 1991), is caused by a paramyxovirus. The paramyxovirus contains a single stranded, negative-sense RNA molecule that encodes six proteins. Two of these proteins, the haemagglutinin neuraminidase and fusion glycoproteins.are antigenically important,

The first recorded outbreak of ND in South Africa occurred in 1946. However, sporadic outbreaks of ND still occur in commercial poultry vaccinated against the virus. The causative agents of these outbreaks have only been identified as NDV, and no strain differentiating techniques have been used to determine neither the identity nor the relatedness between the various aetiological agents. Physicochemical tests, plaque formation assays have been explored by various researchers to try to group these viruses into epidemiologically meaningful groups. Limited success in grouping these viral strains was achieved with the advent of mAbs. An improvement in the specificity of the groups resulted when an RT-PCR based detection method was used to group NDV isolates into distinct genotypes by Ballagi-Pordány and co-workers in 1996.

This technique has the advantages of being quick, sensitive and easy to perform. Its limitation is that it requires specialized knowledge of the techniques employed. This technique will be used to survey 1998 NDV isolates obtained from various sectors of the poultry industry in South Africa.

2.2 Clinical Signs of Newcastle Disease

Newcastle disease virus produces a disease with varied clinical severity and transmissibility (Marin *et al.*, 1996). Three main body systems the respiratory tract, intestinal tract, and nervous

systems are targeted by the virus. The first clinical signs associated with ND are an unusual disinterest in food and water throughout the entire flock (Sainsbury, 1992; Alexander *et al.*, 1992).

Clinical signs associated with the respiratory system include respiratory distress (Alexander, 1991), bubbling, gurgling, choking (Kaschula *et al.*, 1946), and weird-high pitched squawking sounds during respiration (Sainsbury, 1992; Kaschula *et al.*, 1946). The latter symptom seems to be unique to NDV, although it has only been reported in isolated cases (Kaschula *et al.*, 1946). Sneezing (Kaschula *et al.*, 1946), diphtheritic laryngitis, and small cheesy deposits on the congested mucosa of the pharynx (Kaschula *et al.*, 1946), are indications of infection in the respiratory tract.

Infection in the intestinal system is detected by a watery secretion (Pienaar & Cilliers, 1987), and diarrhea (Alexander, 1991), often green in colour (Sainsbury, 1992; Kaschula *et al.*, 1946). Symptoms associated with the intestinal system do not vary greatly, and amount to the few noted above.

In the nervous system, infection is indicated by the presence of torticollis, and paralysis of the wings (Lemahieu *et al.*, 1985; Sainsbury, 1992). Deformation of the feathers (Lemahieu *et al.*, 1985), depression (Alexander, 1991), and incoordination are visible signs that reflect its effect on the nervous system. Extension of the head and neck during inhalation and retraction during exhalation have also been observed in infected poultry (Kaschula *et al.*, 1946). Of the three main agents responsible for respiratory diseases in commercial broiler chickens, Newcastle disease virus (NDV), Infectious bronchitis virus (IBV), and Infectious laryngotracheitis virus (ILTV) (Lockaby *et al.*, 1993), NDV is the only virus within this group, which results in nervous signs (Sainsbury, 1992).

Clinical symptoms produced in other body parts are, necrosis on the tip of the tongue for a distance of approximately 5mm in diameter, which later falls off. This particular phenomenon has only been reported by Kaschula and co-workers in 1946. The presence of excess mucous in the mouth and a thick mass thereof hanging from the beak, a dull appearing cornea, a vacant

stare in the eyes with a comb that darkens as the disease progresses, have been reported (Kaschula *et al.*, 1946).

Death occurs 5-6 days after the appearance of symptoms in poultry (Kaschula *et al.*, 1946). Cessation of egg production, oedema of the head, face, and wattles, are also some of the generally occurring symptoms indicative of NDV infection (Alexander, 1991).

For each particular case, some, all, or none of the above mentioned clinical signs may be present. The previously mentioned clinical signs are non-pathognomonic, as is the case with other avian paramyxoviruses (Alexander, 1991). In contrast, the presence of symptoms in these three systems is thought to be indicative of ND (Sainsbury, 1992). This may result in confusion with other diseases that produce similar symptoms, therefore diagnosis requires further investigation (Alexander, 1991). Although serology provides an indication of the aetiological agent, diagnosis must be confirmed with isolation, identification, and characterization of the infecting strain (Alexander, 1991). Diagnostic differentiation between all possible agents has largely been based on virus isolation from fresh tissue and demonstration of increased serum antibody titres, as gross and microscopic lesions produced can be masked by concurrent infection with other viruses and bacteria, and by the effects of environmental ammonia (Lockaby *et. al.*, 1993).

The incubation period normally persists for 2-7 days in poultry although it may be prolonged to 3 weeks (Sainsbury, 1992). Shedding of the virus is limited and no carriers have been reported to date (Kaschula *et al.*, 1946).

The disease produced following infection is a product of both the infecting strain and host (Alexander, 1991). Other factors pertinent to the effect of infection include, the species of bird, host age, host immune status, presence of exacerbating organisms, adverse environmental conditions (Alexander, 1991), challenge dose and route, virulence of the field strain, other concurrent infections e.g. Mycoplasma, nutritional status, and other stressors (Verwoerd, 1997). Other commonly described indicators include; body temperatures exceeding 109°F, a 95% mortality rate of those infected, a varied morbidity in recovered birds with different forms of paralysis of the wings, legs, and neck (Kaschula *et. al.*, 1946).

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2.3 The classification and description of paramyxoviruses

2.3.1 Description of paramyxoviruses

Newcastle disease virus belongs to the Paramyxovirus genus. Paramyxoviruses contain RNA as their genetic material, packaged in a ribonucleoprotein core that is surrounded by a membrane (Choppin & Compans, 1975).

The RNA molecule consists of 15,586 nucleotides (Phillips *et al.*, 1998). The RNA codes for six genes in the following 3'-5' direction: nucleoapsid protein (NP), phosphoprotein/phosphorylated nucleocapsid associated protein (P), matrix/membrane protein (M), fusion protein (F), haemagglutinin neuraminidase protein (HN), large polymerase protein/ RNA dependent RNA dependent RNA polymerase (L) (Chambers *et al.*, 1990).

2.3.2 The classification of NDV

Classical classification of NDV

NDV belongs to the Paramyxovirus family of enveloped, (-) stranded RNA viruses (Kingsbury & Darlington, 1968). NDV is also called Type 1 Avian paramyxovirus (A-PMV1) and is the type species of the genus Paramyxovirus, family Paramyxoviridae (Jestin & Jestin, 1991). In contrast, De Leeuw and Peeters (1999), suggest that NDV should be classified in the genus Rubulavirus. Based on the alignments of the entire RNA genome sequences of NDV LaSota and other members of the Paramyxoviridae. These researchers suggest that NDV be placed in a new genus within the subfamily Paramyxovirinae (De Leeuw & Peeters, 1999).

NDV is the first member of the genus Paramyxovirus (PMV) to be isolated from birds in 1926 (Alexander, 1991). Other serologically distinct groups isolated from avian species were discovered from 1956 onwards, but viruses belonging to A-PMV-1 have been found to be the most important pathogens for birds of all types (Alexander, 1991). Viruses

belonging to the group PMV-1 and PMV-2 also cause serious disease problems in domestic poultry (Alexander, 1991).

Serological classification

With the use of conventional serological techniques, it was observed that viruses belonging to the A-PMV-1 serogroup were antigenicaly homogeneous in nature (regardless of virulence or source) and as a result where grouped in the A-PMV-1 serogroup (Alexander *et al.*, 1987). In contrast, antigenic differences were detected between classical viruses, and an A-PMV-1 virus that caused a panzootic in pigeons in the 1980s and 1990s (Alexander *et al.*, 1987).

Differences in the epitopes of isolates grouped in the A-PMV-1 serogroup were observed using mAbs (Abenes *et al.*, 1986; Alexander *et al.*, 1985; 1987; Erdie *et al.*, 1987; Hoshin *et al.*, 1983; Jestin *et al.*, 1989; Lama *et al.*, 1988; Meulemans *et al.*, 1987; Nishikawa *et al.*, 1983; Russell & Alexander, 1983; Srivivasappa *et al.*, 1986).

The Baltimore classification

Viruses were grouped into six distinct groups, based on the mode of gene replication and expression (Dimmock & Primrose, 1994). Paramyxoviruses in this case were grouped into Class V, composed of viruses with a single stranded (ss), (-) sense RNA genome complementary in base sequence to mRNA (Dimmock & Primrose, 1994). In this group gene expression proceeds by the transcription of two or more monocistronic mRNAs from a polycistronic template (Dimmock & Primrose, 1994).

Table 2.1. Avian paramyxovirus serotypes (Alexander, 1991)

Prototype Strain	Main Hosts	Reported natural infections of chickens and/or turkeys
PMV-1/Newcastle disease virus	Various	Very common, worldwide distribution, very severe to unapparent disease
PMV-2/chicken/California/Yucaipa/56	Passerines &turkeys	Common, probably worldwide, mild respiratory disease unless exacerbated
I PMV-3/turkey/Wisconsin/68	Turkeys	Turkeys only, in North America and Europe, mild respiratory disease, and egg production problems
II PMV-3/parakeet/Netherlands/75	Psittacines, passerines (Pet birds only)	None
PMV-4/duck/Hong Kong/199/77	Waterfowl	None
PMV-5/budgerigar/Japan/Kumitachi	Budgerigars	None
PMV-6/duck/Hong Kong/199/77	Ducks, geese	Respiratory disease and egg losses in turkeys
PMV-7/dovr/Tennessee/4/75	Pigeons doves	None
PMV-8/goose/Delaware/1053/75	Ducks, geese	None
PMV-9/duck/New York/22/78	Ducks	None

Viruses belonging to the avian paramyxovirus type 1 serogroup, along with eight other A-PMV serotypes have been placed in the genus Rubulavirus, Sub-family Paramyxovirinae, order Mononegavirales (Rima *et al.*, 1995). This classification of NDV is in accordance with that stated in the Sixth ICTV Report.

2.4 The morphology and structure of the Newcastle disease virion

Newcastle disease virus as a typical paramyxoviruses is composed of a membrane containing envelope covered with surface projections, and a helical ribonucleoprotein (RNP) nucleocapsid enclosed within the envelope (Choppin & Compans, 1975).

The virion particles vary in size, between 150-200nm in diameter and are roughly spherical in shape, although very large pleomorphic virions thought to be filamentous forms, distorted under EM preparative procedures and staining, have been observed (Choppin & Compans, 1975; Compans *et al.*, 1966, Howe *et al.*, 1967).

2.4.1 Virion Proteins

A study of purified preparations of SV5, a member of the paramyxovirus genus, showed that this virus contained 0.9% RNA, 0.1% CHO, 20% lipids, and 73% protein (Klenk & Choppin, 1969). Similar results were obtained with NDV by Nakajima & Obara (1967). As mentioned, NDV contains six polypeptides each of which is coded for by a single gene. Early studies predicted 3 major proteins and a variable number of other proteins (Evans & Kingsbury, 1969; Haslam *et al.*, 1969; Bikel & Duesberg, 1969). Below is a table of the known paramyxovirus proteins and their suggested designations and functions (Table 2.2).

Table 2.2 Summary of the properties of some paramyxovirus proteins and suggested designations (Choppin & Compans, 1975)

Suggested designation	M Wt.	CHO presence	Function
P	69 000	-	In virion RNA polymerase complex
HN	67-74 000	+	Haemagglutinin, neuraminidase
F0	65 000	+	Precursor of F protein
NP	56-61 000	-	Nucleocapsid subunit
F	53-56 000	+	Cell fusion haemolysis
M	38-41 000	-	Membrane protein

2.4.2 NDV matrix protein

The viral envelope is derived from the host cell plasma membrane, and contains three viral proteins a nonglycosylated membrane protein (M), and two glycoproteins the haemagglutinin neuraminidase (HN), and the fusion protein (F) (Bratt & Hightower, 1977; Rott & Klenk, 1977).

Matrix protein structure and the function of the different protein motifs

The matrix protein is encoded by an mRNA molecule of ~1200 bases excluding the poly A tail (Wilde & Morrison, 1984). In 1987, McGinnes and Morrison reported that the translational product of this mRNA molecule is a 39 742 Da protein, in agreement with

previously determined molecular weights of 39 000 Da (Collins *et al.*, 1978). The protein is predicted to contain at least two domains, one of which anchors it into the lipid bilayer, the other interacts with the nucleocapsid structure of the core (Ogden *et al.*, 1986).

The matrix protein is located beneath the viral lipid bilayer, and evidence exists which indicates that paramyxovirus M proteins interact with viral glycoproteins (Tyrrell & Ehrnst, 1979). It has been proposed that a critical interaction exists between the M protein and fusion protein in order to allow the formation of infectious virions (Peeples & Bratt, 1984). It is also suggested that the M protein interacts with the viral membrane and viral nucleocapsid. Kyte & Doolittle, (1982), however, failed to locate a hydrophobic region implicated in the interaction with the lipid bilayer. In contrast, McGinnes & Morrison, (1987) report the location of a stretch of 12 neutral hydrophobic amino acid residues beginning 55 residues from the amino terminus.

Similar to the M protein of Rhabdoviruses and other Paramyxoviruses, the NDV M protein sequence was found to code for a number of paired basic amino acid residues i.e., Arg-Arg, Lys-Lys, Lys-Arg, Arg-Lys (McGinnes & Morrison, 1987). NDV in particular was found to contain eight pairs of basic residues, five in the carboxy terminal half of the molecule (McGinnes & Morrison, 1987). The role of these paired basic amino acids in the M protein function is yet unclear. It is thought however, that they may be important in binding the protein to the ribonucleocapsid core of the virus (McGinnes & Morrison, 1987).

Matrix protein Functions

The M protein plays a vital role in the assembly and budding of virions (Rott & Klenk, 1977). Due to its location beneath the viral envelope, it is thought that through its interactions with the envelope, it could play a crucial role in maintaining the structure and integrity of the viral envelope (Choppin & Compans, 1975). It could also be the location of an unknown mechanism which excludes host proteins from areas of the membrane which become the viral membrane by allowing the migration of host cell proteins.

The matrix protein is also proposed to function as the recognition area for viral nucleocapsids during the alignment of nucleocapsids beneath areas of the cell membrane containing viral membrane proteins (Choppin & Compans, 1975).

Paramyxoviridae undergo genome replication, mRNA transcription, protein synthesis, and the assembly of viral components in the cytoplasm (Coleman & Peeples, 1993). However, the matrix protein of NDV is primarily located in the nuclei of infected cells (Hamaguchi *et al.*, 1985), unlike other NDV proteins found in the cytoplasm. NDV M is detected in the nucleus early in infection, and is found concentrated in the nucleoli and remains concentrated in this region throughout infection (Coleman & Peeples, 1993). This localization occurs in many cell types infected with NDV, therefore suggesting that localization occurs via a mechanism common to many cell types (Coleman & Peeples, 1993). Other viral proteins were demonstrated not to be required for nuclear localization of M proteins (Peeples & Bratt, 1982).

NDV M proteins have two regions resembling two types of nuclear localization signals (NLSs) on either end of a 17 amino acid region (residues 246-263), two highly basic amino acid clusters in which four out of the five amino acids are Arg (R) or Lysine (K) (Coleman & Peeples, 1993). A second region (amino acid residues 252-364) resembles a bipartite NLS. It contains two pairs of basic amino acids, separated by nine amino acids, and is located at the extreme carboxy terminus of the M protein (Coleman & Peeples, 1995). These results indicate that the carboxy terminal motif is not involved in nuclear localization (Coleman & Peeples, 1993). Both basic clusters located between amino acids 247 and 263, cluster I (amino acids 247-258; cluster II amino acids 259-263), are required for nuclear localization of the M protein (Coleman & Peeples, 1993). The M protein NLS was demonstrated to be bipartite (Coleman & Peeples, 1993). The secondary structure of which is predicted to be α-helical (Garnier, 1978; Coleman & Peeples, 1993). The biological significance of the NLS region of NDV M protein is unknown. It is postulated that due to its role in viral assembly, its enclosure in the

nucleus may be a strategy for limiting its cytoplasmic concentration, thus enabling viral transcription whilst down-regulating budding (Coleman & Peeples, 1993).

Comparison of NDV M protein with that of other Paramyxoviruses

A comparison of NDV M protein sequences with that of available paramyxoviruses M protein sequences shows a lack of homology (McGinnes & Morrison, 1987). Similarities in the range of 35-37% were observed between members of the paramyxoviridae (McGinnes & Morrison, 1987). The positions of the paired basic residues are well conserved upon comparison of SV and NDV M protein sequences, suggesting their importance in maintaining M protein structure and function (McGinnes & Morrison, 1987).

2.4.3 NDV glycoproteins

Three main patterns of glycoprotein expression on viral envelopes have been differentiated amongst NDV strains using polyacrylamide gel electrophoresis (PAGE):

(Klenk *et al.*, 1977a).

HN	(MW. 74 000d)	
F	(MW. 56 000d)	Observed in virulent strains (Italien & Herts)
HN	(MW. 74 000d)	
F	(MW. 68 000d)	Found in avirulent (mesogenic) strains (LaSota, B1, F)
HNo - HNo	(MW. 82 000d)	
Fo	(MW. 68 000d)	Seen in avirulent strains (Ulster, Queensland)

It is thought that virulent and avirulent NDV strains have evolved from each other by spontaneous mutation (Klenk *et al.*, 1977a). This event caused a change in the susceptibility of viral glycoprotein precursors to proteolytic activation (Klenk *et al.*, 1977b). Susceptibility of glycoproteins to proteolytic activation is a structural phenomenon and not a consequence of the level of proteases in certain infected cells (Nagai *et al.*, 1976). These glycoproteins play important roles that determine the virulence of different strains of NDV.

2.4.3.1 The fusion glycoprotein

The fusion glycoprotein allows fusion of the viral envelope and cell membrane that precedes penetration of the viral RNA (Klenk, *et al.*, 1977b), and haemolysis (Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Seto *et al.*, 1981).

It has become clear that the level of infection produced by a particular strain will not only be dependent upon the size of the inoculum, but will also be affected by the occurrence of the above events (Klenk, *et al.*, 1977b). These in turn depend on the susceptibility of these glycoproteins to proteolytic activation. Thus it can be assumed that infection with a virulent strain producing biologically active progeny in a wide range of different cells, spreads more rapidly in the organism than infection with an avirulent strain which will have a narrow host range and may only undergo the first cycle of replication in certain tissues of the host (Klenk, *et al.*, 1977b).

The fusion glycoprotein of NDV mediates fusion between the plasma membrane of infected cells and uninfected cells and between the membranes of the host cells with the virion envelope (Bratt & Gallagher, 1969; Mountcastle *et al.*, 1971; Nagai *et al.*, 1976). This protein is synthesized as a precursor (F0) (Sampson & Fox, 1973). F0 is subsequently cleaved into disulphide linked F1-F2 (Scheid & Choppin, 1977). F0 is the inactive fusogen until cleavage has occurred (Nagai *et al.*, 1976; Schwalbe & Hightower, 1982; Scheid & Choppin, 1974). A host cell enzyme present in some, but not all host cells is responsible for the cleavage activation of F0 (Rott & Klenk, 1977). Unlike that of Sendai virus (another paramyxovirus), it has been reported that the cleavage of the NDV fusion glycoprotein occurs intracellularly (Nagai *et al.*, 1976; Seto *et al*, 1981).

The location of the fusion glycoprotein on the paramyxoviruses virion particles.

This glycoprotein is present covering the virion surface as spikes 8-12nm in length (Rott & Schäfer, 1961; Hosaka *et al.*, 1961; Choppin & Stoeckenius, 1964). The entire glycoprotein is accessible to protease treatment and does not penetrate deeply into the envelope bilayer (Chen *et al.*, 1971). Removal of fusion glycoprotein spikes does not

disrupt the virion, implying that it does not play a role in maintaining viral membrane structural integrity (Chen *et al.*, 1971).

Fusion glycoproteins of paramyxoviruses can be classified as intergral membrane proteins (Rott & Klenk., 1977). The fusion protein is anchored in the viral membrane at its carboxyl terminus in a standard fusion (Schaper *et al.*, 1988). It however, has no apparent role in the maintenance of structural integrity of the viral membrane (Chen *et al.*, 1971).

Structure of the Fusion glycoprotein

The fusion glycoprotein of NDV is encoded by an mRNA molecule of ~1759 bases (McGinnes & Morrison, 1986). The mRNA molecule translates into a 553 amino acid long polypeptide of molecular weight 58 978D for the unglycosylated, uncleaved form of the protein (Espion *et al.*, 1987).

The fusion protein contains a signal sequence (residues 9-25) composed of 17 hydrophobic uncharged amino acids at its N-teminus (Toyoda *et al.*, 1987, McGinnes & Morrison, 1986). This hydrophobic core is suggested to serve as a signal sequence for the insertion of the protein into the membrane of the rough endoplasmic reticulum (Toyoda *et al.*, 1987). It is thought to act as a signal sequence for protein translocation across membranes (von Heijne, 1983). Three potential cleavage sites have been observed at positions 22, 24 and 25 (Ala, Gly, and Lys respectively), (Toyoda *et al.*, 1987).

The hydrophobic core is located within the N-terminal region (residues 2-32) of the fusion protein (Toyoda *et al.*, 1989). The N-terminal end of the protein was observed to be highly variable, with differences of up to 45 % between some NDV strains (Toyoda *et al.*, 1987, Toyoda *et al.*, 1989). A cluster of 27 hydrophobic uncharged amino acids (residues 500-522) at the C-termius end form the transmembrane anchorage domain (Toyoda *et al.*, 1987; Toyoda *et al.*, 1989). This region is followed by the cytoplasmic tail region composed of 27 relatively hydrophilic residues (residues 523-553), (Toyoda *et al.*, 1987; Toyoda *et al.*, 1989; McGinnes & Morrison, 1986). The cytopalsmic tail is

thought to play a possible role in the transport of glycoproteins and may interact with other viral proteins during viral assembly (Toyoda *et al.*, 1989). Amino acid sequences of the membrane anchoring and cytoplasmic domains of different NDV strains showed homologies of 91.2-99.8% (Toyoda *et al,* 1989). These regions are less variable than the N-terminal region, and this is thought to be indicative of the structural and functional constraint exerted on the change of the whole fusion glycoprotein molecule (Toyoda *et al.*, 1989).

The fusion cleavage site is located at residues 112-116 (Toyoda *et al.*, 1987; Toyoda *et al.*, 1989). It serves as the recognition site for the proteolytic cleavage of F0 (F precursor protein), into F1-F2 disulphide linked sub-units (Nagai *et al.*, 1976; Scheid & Choppin, 1977). The hydrophobic, fusogenic region is located adjacent to the fusion cleavage site (residues 117-142), (Toyoda *et al.*, 1989; Toyoda *et al.*, 1987). This F1 N-terminus of the proteolysis generated F1 sub-unit is implicated in the membrane fusion reaction, and its amino acid sequence is similar to that previously reported for the Hickman strain of NDV (Richardson *et al.*, 1980).

The fusion proteins of paramyxoviruses are anchored in the membrane by its C-terminal region (Toyoda *et al.*, 1989). Comparison of the total amino acid sequence in this region of 11 NDV strains showed a high degree of homology (89.3-99.6%) in pairwise comparison (Toyoda *et al.*, 1989).

Eight potential glycosylation sites (Asn-X-Ser/Thr) are strictly conserved, with exceptions at residues 192-194, 497-499 in strain MIY/51 (Toyoda *et al.*, 1989). The first site (res. 85-87) is located in the F2 sub-unit (McGinnes & Morrison, 1986). If it is assumed that the signal sequence and fusion cleavage sites are removed, then F2 will have 85 amino acid residues and F1 437 (McGinnes & Morrison, 1986; Toyoda *et al.*, 1989). In 1977, Scheid and Choppin demonstrated that the ratio of glucosamine to amino acid-labeling was similar in F1 and F2 giving an approximate ratio of 1:5. It can therefore be assumed that five of the seven potential glycosylation sites in F1 are glycosylated (Toyoda *et al.*, 1987; Toyoda *et al.*, 1989; McGinnes & Morrison, 1986). Site 541-543 may not be glycosylated due to it being located in the probable cytoplasmic

tail (McGinnes & Morrison, 1986). The other sites located either at residues 191-193, 192-194 may be unglycosylated as a result of spatial constraints not enabling each to accept an oligosaccharide chain of 2000-3000 (Toyoda *et al.*, 1989; McGinnes & Morrison, 1986).

Nine cysteine residues are completely conserved in paramyxovirus fusion proteins (Toyoda *et al.*, 1987) except at residues 27, 199, 347, 410, and 514. These include Cys residue 76 that forms the disulphide linkage between F1 and F2 subunits (Toyoda *et al.*, 1987; Toyoda *et al.*, 1989). Inclusive of Cys 76, another cysteine residue occurs in F2 located in the presumed signal sequence (Toyoda *et al.*, 1989). The F1 subunit contains a cluster of seven cysteine residues located between residues 338-424 (McGinnes & Morrison, 1986). These residues are implicated in intrachain disulphide bonds within F1 that cause folding of the polypeptide (Toyoda *et al.*, 1989). The other two cysteines are located in the membrane anchorage domain (McGinnes & Morrison, 1986; Toyoda *et al.*, 1989). Their location in this area has led to suggestions that they may not be utilized for disulphide bond formation, but for fatty acid acylation. It has been suggested that the conservation of Cys residues in the C terminal half of the molecule may be important in maintaining the structural framework characteristic of paramyxovirus fusion proteins (Toyoda *et al.*, 1987).

The F1 N-terminal sequence is hydrophobic and contains spaced glycines (Toyoda *et al.*, 1989). The sequence Phe-X-Gly at the N-terminus end is highly conserved among paramyxoviruses (Espion *et al.*, 1987). The Phe (+1) aromatic residue at the F1 N-terminus, although not essential for paramyxovirus fusion, appears to be structurally conserved and functionally important for viral fusion activity (Toyoda *et al.*, 1989). The location of Leu (+1) at this position has however, been observed to be unique to strains with HN glycoprotein that form from a larger precursor HN0

Fusion protein synthesis and post-translational modifications

Fusion protein synthesis and assembly

F0 is an inactive fusogen until cleavage has occurred (Nagai *et al.*, 1976, Schwalbe & Hightower, 1982). Similar to other viral glycoproteins, the fusion protein is synthesized in the rough endoplasmic reticulum (Nagai *et al.*, 1976), transported to the cell surface via host cell pathways, i.e. via the smooth endoplasmic reticulum and Golgi apparatus to the plasma membrane (Nagai *et al.*, 1976). During its transport, it undergoes post-translational modifications (Klenk *et al.*, 1977b). From analogy to influenza virus these include, sequential glycosylation on the rough endoplasmic reticulum (Klenk *et al.*, 1977b; Mountcastle *et al.*, 1971). In addition, it is fatty acid acylated on smooth internal membranes (Chatis & Morrison, 1982). It has also been reported that the fusion protein undergoes a conformational change during intracellular transport that involves the disruption of some intramolecular disulphide bonds (McGinnes *et al.*, 1985).

Activation and viral tropism

The fusion protein undergoes modification by proteolytic cleavage, which occurs at the smooth internal membranes and at the plasma membrane of the larger precursor (F0) to glycoprotein (F) in NDV (Hightower & Bratt, 1975). A host cell enzyme present in some, but not all host cells is responsible for the cleavage activation of F0 (Rott & Klenk, 1977). F0 is cleaved into disulphide liked F1-F2 (Scheid and Choppin, 1977) unlike that of the paramyxovirus Sendai virus, it has been reported that the cleavage of NDV fusion protein occurs intracellularly (Nagai *et al.*, 1976; Seto *et al.* 1981). Cleavage activation has been reported to occur in the trans-Golgi membranes of the infected cell (Morrison *et al.*, 1985; Nagai *et al.*, 1976). Cleavage is directed by trypsin-like proteases (Toyoda *et al.*, 1987).

The amino acid sequence in the cleavage activation site for the virulent NDV strain Australia-Victoria (AV) was determined as: Arg-Arg-Gly-Lys-Arg (McGinnes & Morrison, 1986). This sequence is similar to that of cleavage sites of precursors to peptide hormones. It is well documented that hormone precursors are processed in the trans-Golgi membranes of secreting cells (Steiner *et al.*, 1980). Similarly Morrison *et al.*, (1985) reported that F0 cleavage is located in the trans Golgi membranes. Therefore, in analogy, it can be concluded that NDV-AV also uses that same cellular enzymes used to process hormone protein precursors (McGinnes & Morrison, 1986).

Amino acid sequences at the fusion cleavage site for other paramyxoviruses, NDV, SV5, and RSV have also been found to contain similar residues Arg-Arg-Lys-Arg-Arg.Lys. NDV has a wide range of strains with different pathogenicity for their natural host, the chicken (Klenk *et al.*, 1977a). It is unique amongst paramyxoviruses because significant variation in the virulence has been observed in the same serotype (Waterson *et al.*, 1967).

Proteolytic activation of F0 is important for the fusion of the viral envelope with the target cell membrane and therefore, for initiation of virus infection (Scheid & Choppin, 1977). Cleavage of F0 liberates the N-terminus of F1, which has been shown to be essential for the biological activity of F (Nagai *et al.*, 1976).

NDV F0 has been reported to play an important role in viral tissue tropism and pathogenesis in birds (Nagai *et al.*, 1976). The F0 protein of avirulent NDV strains is not efficiently cleaved in many cell types. Similar to Sendai virus F0 proteins, the haemagglutinin (HA) protein of human influenza viruses have been shown to only contain one Arg residue at the cleavage activation site (Ward & Dopheide, 1980; Gething *et al.*, 1980). The F0 protein of virulent NDV strains Italien, Herts and Miyadera, have the same sequence at their cleavage sites (Arg-Arg-Gln-Arg-Arg) (McGinnes & Morrison, 1986).

The cleavage sites of F0 proteins in avirulent strains LaSota, D_{26}, Ulster, and Queensland had basic residues at positions –2 and –5 substituted for either glycine or serine without exception (Toyoda *et al.*, 1987). Each of the above changes were due to one single base substitution (Toyoda *et al.*, 1987).

It is not clear whether both dibasic residues are required for efficient proteolytic cleavage, as available evidence indicates that one pair of basic residues is the minimum but sufficient requirement for proteolytic processing of hormones and viral proteins in eukaryotic cells (Rice & Strauss, 1981). In contrast, however, it has been observed that in order to yield the correct hydrophobic F1 N-terminus, Arg-Arg at positions –1 and –2 is important (Toyoda *et al.*, 1987)

It has been demonstrated that proteolytic activation of HA influenza proteins is not only a result of simple cleavage of the polypeptide by the endopeptidase, but is accompanied by the removal of the arginine residue by an exopeptidase like carboxypeptidase (Garten *et al.*, 1981). It is thought that a similar removal of the cleavage site of NDV F0 protein may occur as proteolytic activation is paralleled by an acidic shift in the isoelectric point of the fusion protein (Kohama *et al.*, 1981). If it is assumed that signal sequence cleavage removes 22-25 amino acids from the amino terminal end of the protein and that cleavage of F0 to F1-F2 occurs at the Arg-Arg-Gly-Lys-Arg sequence, then the protein portion of F2 is 86-89 amino acids long (McGinnes & Morrison, 1986).

Consensus sequences of amino acids for the virulent strains at the cleavage site reported to date are: RRQK/RR↓F (amino acid residues 112-117) (Glickman *et al.*, 1988). That of avirulent strains is G/RKQK/GR↓L (amino acid residues 112-117) (Glickman *et al.*, 1988).

Sequence analysis of HA genes of a number of influenza strains has shown that those strains with a limited host range with respect to HA cleavage, contain a single Arg at the cleavage site, whilst the less stringent fowl plaque virus HA protein has the sequence Ly-Lys-Arg-Gln-Lys-Arg at the cleavage site (Porter *et al.*, 1979; Gething *et al.*, 1980).

Therefore, by analogy with influenza, it could be deduced that the cleavage site of the fusion protein of nonpathogenic NDV strains differs from virulent strains by the lack of a series of basic amino acid residues (McGinnes & Morrison, 1986). In which

nonpathogenic strains may contain a single Arg residue at the cleavage site (McGinnes & Morrison, 1986).

Comparison of paramyxovirus fusion proteins

It has been reported that considerable sequence conservation at the amino terminus of the F1 glycoprotein between SV5, NDV, and SV occurs (Richardson *et al.*, 1980). Other than the fusion sequence no extensive regions of homology between the four fusion proteins of SV5, NDV, SV, and RSV have been observed (McGinnes & Morrison, 1986). The positions of potential glycosylation sites are not well conserved within the group of paramyxoviruses.

The positions of the cysteine residues are quite conserved, especially among the paramyxovirus subgroup (McGinnes & Morrison, 1986). All three proteins have similar numbers of cysteine residues (NDV=13, SV5=12, Sendai virus=10, and RSV=16). Seven of the cysteines are precisely aligned and four others occur in very similar positions (McGinnes & Morrison, 1986). The other two cysteine residues of NDV and one other in the SV5 sequence occur in the putative membrane spanning region and may therefore not play a role in disulphide bond formation (McGinnes & Morrison, 1986). Conservation of the positions of cysteine residues points to their importance in the conformation of the protein as determined by the intramolecular and possibly intermolecular disulphide bonding (McGinnes & Morrison, 1986). It has already been shown that the nascent fusion protein of NDV contains extensive intramoleular disulphide bonding (McGinnes *et al.*, 1985).

2.4.3.2 The haemagglutinin-neuraminidase (HN) glycoprotein

Structure of HN protein

HN is a type II glycoprotein, that occurs on the surface of virions and infected cells as a tetrameric spike (Markwell & Fox, 1980; Thompson *et al.*, 1987). The gene which encodes this protein is 2000-2002 bases long (Gotoh *et al.*, 1988; Sakaguchi *et al.*, 1989). This homotetramer contains a membrane proximal stalk that supports a terminal globular

domain (Wang & Iorio, 1999). The globular domain contains the receptor recognition site, the neuraminidase (NA), and antigenic sites (Mirza *et al.*, 1983). These two regions comprise the ectodomain of the HN protein. It is thought that the structure of the globular head of HN is similar to the β-sheet propeller structure of influenza virus NA (Varghese *et al.*, 1992, Colman *et al.*, 1993).

The HN protein is 577 amino acid long (Schaper *et al.*, 1988), with a large amino-terminal hydrophobic portion between residues 27-50. This region is thought to act as the membrane anchoring domain (Schaper *et al.*, 1988; Schuy *et al.*, 1984). It contains five predicted N-linked glycosylation sites (Asn-X-Ser/Thr) (Schaper *et al.*, 1988). The cytoplasmic domain is located between residues 1-26 (Sakaguchi *et al.*, 1989).

The specificity of NDV F protein is mediated by determinants defined by the stalk segment in the ectodomain of its HN protein (Deng *et al.*, 1995). Mutations in this region were found to deter the ability of HN to interact with F (Deng *et al.*, 1995). Residues 212-303 comprise a highly conserved region that is found to resemble the sialic acid – binding site of influenza [NA] (Jorgensen *et al.*, 1987).

Comparison of paramyxovirus HNs

Upon comparison of the protein sequences of 13 NDV HN proteins it was observed that the cysteine residues were well conserved, and it is proposed that cys 123 might be involved in the formation of HN dimers (Sakaguchi *et al.*, 1989; Sheehan *et al.*, 1987). The glycosylation sites were also highly conserved amongst this group of strains (Sakaguchi *et al.*, 1989).

It has been observed that there is less conservation in the stalk than in the globular domain (Morrison & Porter, 1991). A short stretch of twenty residues (93-110), in NDV HN is conserved among paramyxoviruses and Pro residue at position 93 is completely conserved (Wang & Iorio, 1999).

The grouping of NDV isolates based on HN size

The different location of stop codons in the HN gene allows the translation of three HN proteins namely, 616, 577, 571. The largest precursor type is designated HNo616, and requires proteolytic conversion in order to become biologically active (Klenk *et al*, 1977b). The other two are active translation products designated HN577, and HN571 (Sakaguchi *et al.*, 1989). The subgroups generated by HN size correlated well with the nucleotide and amino acid sequence differences (Sakaguchi *et al.*, 1989). Distinct lineages A, B, C based on Ks values strictly correlated with the three main groups classified on the basis of the size of the HN protein (Sakaguchi *et al.*, 1989).

Group A comprised strains that are asymptomatic for chicken and non-lethal for chick embryos, B is composed of a heterogeneous group of virulent and avirulent strains with a MDT in the range 50-120hr. Group C is comprised of typically virulent strains with a MDT for chick embryos ~50 hrs (Nagai *et al.*, 1988). These three lineages were also found to correlate with differences in pathogenicity to some extent (Sakaguchi *et al.*, 1989).

Functions of HN

This protein functions to mediate binding to cells containing sialic acid-rich receptors (Schaper *et al.*, 1988). The role of HN in virus pathogenicity is less well defined than that of the fusion protein (Schaper *et al.*, 1988). HN must act in coordination with the fusion protein for attachment and penetraion (Schaper *et al.*, 1988). Through its neuraminidase (NA) activity, it directs the release of sialic acid from soluble and membrane associated polyproteins (Scheid & Choppin, 1973).

2.5 The fusion process

Paramyxovirus infected cells are characterized by the presence of multnucleate syncytia (Choppin & Scheid, 1980). Fusion requires the combined efforts of the HN and F proteins (Lamb, 1993). HN recognizes receptors on the cell surface (Hsu *et al.*, 1979), and the F promotes penetration of target cell membrane (Scheid & Choppin, 1974). Circular dichroism

studies suggest that prior to fusion, the F protein undergoes a conformational change, exposing the fusion peptide and enabling it to mediate penetration of the target membrane (Citovsky *et al.*, 1986). It is postulated that the recognition of receptors by HN triggers this conformational change in paramyxovirus F protein (Lamb, 1993). The interaction of these two glycoproteins is very virus specific (Deng *et al.*, 1995).

Two models have been suggested for the mechanism of fusion that occurs between cells:

1. Syncytium formation results due to the attachment of sialic acid-containing receptors on target cells by the receptor recognition site in the terminal globular domain of the HN (Lamb, 1993). This event induces a conformational change in F converting it to its fusogenic form and initiating fusion (Deng *et al.* 1995).

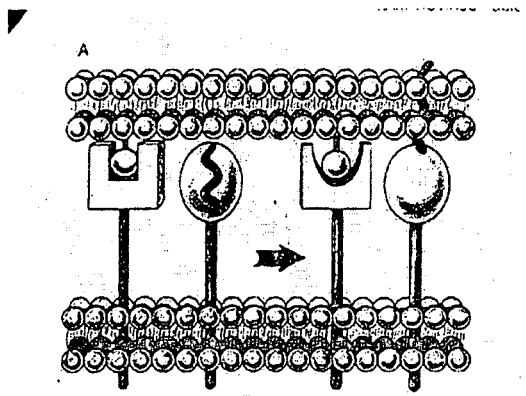

Figure 2.1 Schematic illustration of the hypothesized conformational change in the paramyxovirus F protein to liberate the fusion peptide. HN (rectangular box), F (oval containing the buried fusion peptide) (Lamb, 1993).

2. HN and F interact in the RER, independent of receptor recognition (Stone-Hulslander & Morrison, 1997). This complex maintains the two proteins in a prefusion state (Deng *et al.*, 1995). When HN binds receptors, it triggers a conformational change in F dissociating from it immediately (Deng *et al.*, 1995).

2.6 Development of detection methods for NDV

The typing of viruses is important for the characterization of viral populations and in order to study their epidemiology (Charrel & Chandler, 1999). Historically serological methods were used for typing i.e., identifying antigenic differences among virus populations (Charrel & Chandler, 1999). Laboratory tests done routinely e.g. virus isolation and/ or serological testing using HI tests (Sainsbury, 1992), are efficient at detecting the presence of NDV specific antibodies. However, they do not generate results that will enable epidemiological tracing of strains during outbreaks. The above mentioned tests have a role in diagnosis, that of the identification of disease agents in the field. Their disadvantage is that they do not provide comprehensive, qualitative, and detailed information about a particular strain of interest, as compared for example with mAb and nucleic acid probes (Cavanagh, 1985).

Serology

Haemagglutination and haemagglutination inhibition tests are an indirect method of viral detection. HI tests are the accepted method for detecting NDV-specific antibodies in poultry sera (Alexander, 1991). This technique has the shortfall that non-specific reactions may be induced by sera from other species (Koch *et al.*, 1998).

Serotype differentiation is routinely part of the initial diagnosis and uses polyclonal antisera to the prototype strains of avian paramyxovirus serotypes in HI tests (Alexander, 1991). This technique requires specific laboratory conditions and skilled personnel (Thirumurugan *et al.*, 1997). It is time consuming and takes upto three weeks or longer for definite diagnosis (Verwoerd, 1997). Serological techniques are accompanied by conventional virological methods which have the limitations of being slow, expensive, and requiring specialists infrastructure (Verwoerd, 1997).

Pathogenicity Tests

A battery of tests which include MDT, ICPI, IVPI, Intracloacal Inoculation Pathogenicity test allow for the grouping of NDV isolates into five pathotypes, viscerotropic velogenic NDV, neurotropic velogenic NDV, mesogenic NDV, lentogenic NDV, and asymptomatic NDV

(Alexander, 1991). The Intracloacal Inoculation Pathogenicity test also has the added advantage of differentiating viscerotropic velogenic NDV from other strains (Beard & Hanson, 1984). These tests are disadvantageous as they are time consuming, involve animal experiments and are relatively costly (Verwoerd, 1997). Many problems have been encountered using serological method for diagnostics, as these techniques do not differentiate between vaccine strains and pathogenic field strains.

These limitations have resulted in the development of techniques that confer advantages of rapid detection of small quantities of pathogens in small quantities of bodily fluids, biopsy or post-mortem materials (Cavanagh, 1985).

ELISAs

Recently ELISAs have been developed for the detection of antibodies (Ab) for avian diseases. This technique is easily automated and results can be obtained rapidly and inexpensively (Alexander, 1991). The results correlate well with HI results, are reproducible, highly specific and sensitive and allow the distinction between protective and non-protective (Ab) levels (Alexander, 1991).

Improvements on this method led to the development of the Dot-enzyme, and Avidin Biotin Dot ELISAs. These techniques are highly specific (Thirumurugan *et al.*, 1997), rapid, simple efficient and do not require trained personnel (Thirumurugan *et al.*, 1997). The Dot-Blot ELISA has been demonstrated to be more sensitive than the HI in detecting NDV antibodies after infection (Folitse *et al.*, 1998). These tests however are costlier than the routinely performed HA/HI tests (Thirumurugan *et al.*, 1997).

Latex Agglutination Test

This technique is rapid, simple and does not require trained personnel for its operation (Thirumurugan *et al.*, 1997). It allows on site NDV detection and the NDV Ab coated beads were shown to be stable at 37°C for five weeks (Sreedevi *et al.*, 1997).

Immunoperoxidase test (IPT)/ Immunohistochemical tests

IPT allows for the rapid detection of different antigens (Lockaby *et al.*, 1993). Trained personnel are however required for the production of formalin-fixed tissues (Lockaby *et al.*, 1993).

Ease of interpretation of the specific stain, limited possibilities of non-specific staining, negligible background staining, and efficient staining are some of the advantages of this technique (Lockaby *et al.*, 1993). The following limitations have been reported in literature, potential degradation of Ag sites by formalin fixation, tissue processing, positive staining restricted to respiratory epithelium, and limited sensitivity (Lockaby *et al.*, 1993).

Complement Fixation

Complement fixation is another test applied for the rapid identification of NDV isolates. It allows the differentiation of velogenic field isolates from vaccine strains (Butterfield & Graves, 1974). It has the added advantage of a short incubation period and only requires visual reading for interpretation of results (Butterfield & Graves, 1974).

RNA Oligonucleotide Fingerprinting

This technique does not require extensive nucleic acid sequencing, and describes distinguishing characteristics among velogenic NDV strains that assist in their identification (Palmieri & Mitchell, 1991). It has the disadvantage of the absence of easily identifiable large "marker" oligonucleotides characteristics of NDV strains (Palmieri & Mitchell, 1991). Another shortcoming of this technique is that only 10-15% of the viral genome can be visualized (Palmieri & Mitchell, 1991).

Oligonucleotide Probes/ Slot-Blot Hybridization

With the advent of nucleic acid sequencing it became apparent that certain regions within the NDV genome were highly conserved. Nucleic acid probes for the 5' non-coding region of the fusion protein gene, highly conserved within classes of negative-stranded RNA viruses (McGinnes & Morrison, 1986) were used to detect NDV (Jarecki-Black & Palmieri, 1992). The hybridization assays used are easy to perform, require minimal amounts of RNA and enable

rapid screening of up to 24 samples per blot (Jarecki-Black & Palmieri., 1992). This technique is limited in its ability of differentiate between vaccine strains and velogenic field strains (Jarecki-Black & Palmieri., 1992). It can be used for the screening of RNA from a variety of sources, identifies all NDV strains, and is capable of distinguishing NDV from other avian disease agents (Jarecki-Black & Palmieri., 1992).

NDV-CL, a probe specific for the fusion protein cleavage sequence of NDV fusion protein was subsequently developed (Jarecki-Black & King, 1993). It hybridized to velogenic and mesogenic NDV strains only (Jarecki-Black & King, 1993).

For a more qualitative investigation into the nature of a particular NDV strain, only two techniques have been described that allow the grouping of NDV isolates into epidemiologically meaningful groups. They are mAb detection inclusive of ELISAs, and an RT-PCR detection technique coupled to sequencing and restriction enzyme analysis

2.7 Methods of Epidemiological surveys of NDV

MAb technology

The specificity of mAb to single antigenic epitopes, have been employed to show similarities or differences between various virus isolates (Alexander, 1991), which appear to be identical using conventional antisera. These mAb have been produced to allow differentiation of strains using a panel of mAb that produce a reaction "fingerprint"(Alexander, 1991). This technique has allowed efficient grouping of viruses sharing biological and epizootiological properties (Alexander, 1991; Alexander *et al.*, 1997).

Employing a panel of twenty-six mAbs Alexander and co-workers (1997), observed 14 different binding patterns for NDV isolates (Alexander *et al.*, 1997). It was established that viruses grouped by the same pattern were either biologically, temporally or geographically related (Alexander *et al.*, 1997). In a previous study performed by Russell and Alexander (1983), two viruses placed in group F were reported to bind to mAb 445. In contrast, Alexander and co-workers (1997) report in a more recent study the binding of this isolate to the mAb 445. It seems

that with an increasing number of isolates studied and an extension of the number of mAbs used an increment in the number of binding patterns occurred as compared to that previously reported of eight patterns (Russell & Alexander, 1983; Alexander *et al.*, 1997).

Using the extended 26 mAB panel an isolate previously placed in group F due to the lack of reactivity with mAb688 was found to bind to it at high concentrations (Alexander *et al.*, 1997). In this study previous group C isolates were divided into two groups C1 and C2, with group C2 isolates showing distinct virulence, host range, and binding differences to those placed in C1 (Alexander *et al.*, 1997).

Binding pattern E included Hitchner B1 and LaSota laboratory and vaccine strains. It included a large number of isolates that are thought to reflect their re-isolation from areas where they were being used as live vaccines (Alexander *et al.*, 1997).

The mAb patterns produced with various NDV strains were found to be homogeneous with respect to virulence for chickens (Alexander *et al.*, 1997). Contrary to the majority of published literature on antigenic stability of the F protein, differences in antigenic stability of the epitopes have been observed (Panshin *et al.*, 1998). Differences were found not only between different sites but also within epitopes belonging to the same site (Panshin *et al.*, 1998).

It is important to note that grouping and clustering based on mAb binding patterns has no phylogenetic significance as a single nucleotide difference may be the cause of such variation (Alexander *et al.*, 1992). The best use of this technique in an epizootiological context is as a tool with which similarities and differences between viruses isolated during outbreaks can be established (Alexander *et al.* 1997; Alexander *et al.*, 1987; Alexander *et al.*, 1992). It has been successfully applied to identify an antigenically variant avian paramyxovirus (APMV) type 1 isolate from pigeons, and allowed its tracing to food stores containing the virus (Alexander *et al.*, 1985)

One of the shortcomings of this technique is the lack of markers that will be able to differentiate vaccine-like strains from indigenous strains of low virulence (King & Seal, 1998). The failure of

two viral isolates to bind to a particular mAb does not mean that they are antigenically identical at that epitope, the apparent antigenic relatedness could be circumstantial (Alexander *et al.*, 1997).

RT-PCR based detection methods

RT-PCR (reverse transcription polymerase chain reaction), was used to amplify a 75% region of the F gene in over 200 strains of NDV (Ballagi-Pordány *et al.*, 1996). Amplicons were digested using three restriction enzymes and the cleavage sites mapped. This technique allowed the grouping of isolates into six major NDV genotypes, and the production of unique fingerprints of vaccine strains (Ballagi -Pordány *et al.*, 1996).

Briefly these groups are as follows:

I: Lentogenic strains mainly from waterfowls, and old chicken isolates prior to 1960.

II: North American isolates with varying virulence including lentogenic and mesogenic vaccine strains

III: Two early isolates from the Far East

IV: European strains from the first panzootic that started in the late 1920s, and their descendents.

V: Strains originating from imported psittacines and the chicken epizootic in 1970

VI: Strains from the Middle East isolated in the 1960s and later isolates from Asia and Europe.
It contains a distinct subgroup of pigeon paramyxovirus-1 strains responsible for the 3rd panzootic

VIa strains from the second panzootic

VIb pigeon PMV-1 strains

VIc strains from Hong Kong and China isolated in the late 1970s , and those from Hungary in the early 1980s.

(Ballagi-Pordány *et a.*, 1996).

The genotypes were found to have a good correlation with mAb binding patterns previously described by Toyoda *et al.*, 1989. This method was used to screen isolates from outbreaks between 1992-1996 in Western Europe (Lomniczi *et al.*, 1998). The strains isolated were grouped in genotypes VI, and VII (Lomniczi *et al.*, 1998).

The advantages of this technique are, the speed at which it can be performed, its reliability for the differentiation and identification of NDV strains (Ballagi -Pordány *et al.*, 1996). Restriction site analysis is a useful tool for screening large numbers of isolates and grouping them into closely related groups (Lomniczi *et al.*, 1998). It however, has the limitations that it cannot provide information regarding the genetic relationships between the groups (Lomniczi *et al.*, 1998).

Table 2.3 Groupings of NDV strains by different methods (Ballagi-Pordány *et al.*, 1996)

Origin of Strains	Virulence in chickens	Antigenic groups by mAbs	Lineages HN and F gene sequences	Genetic groups restriction sites
North America prior	L	E	B	II
to the 1960s	M,V	D	B	II
1st Panzootic (until the 1960s)				
a) Far East	V	B	C	III
b) Europe	V	B	C	IV
2nd Panzootic (From the early 1960s)				
a) Psittacines	V	A	-	V
b) Chicken	V	B, C1	-	Via/c
3rd Panzootic (From the late 1970s)				
Pigeon PMV-1	L, M, (V)	P	-	Vib
Others				
1. Chicken				
F vaccine	L	F	-	II
H vaccine	M	B	-	III
V4, Ulster	L	G	A	I
b) Waterfowl	L	G	A	I
	L	H	-	-
	L	L	-	-
	L	C2	-	-

2.8 Epidemiology of Newcastle disease in South Africa

Nationally recorded ND outbreaks

According to the literature surveyed, the first recorded outbreak of Newcastle disease in South Africa occurred in May 1945, in Natal (now Kwazulu Natal Province) (Kaschula *et al.*, 1946). The outbreak started in Durban towards the end of 1945, and diagnosis was confirmed by the ability of the sera collected to inhibit agglutination of red blood cells (RBCs) (Kaschula *et al.*, 1946). There is no scientifically published literature on NDV outbreaks in South Africa, hence the reliance on newspapers.

It is thought that the infection was introduced through the ports by ships coming in from the East Coast of Africa (Kaschula *et al.*, 1946). The autopsy findings and symptoms described closely resembled those described by Hudson in Kenya (Kaschula *et al.*, 1946).

A subsequent ND outbreak also occurred in Durban (Kwazulu- Natal province) in 1978, killing thousands of birds in the region (Natal Mercury: 2/10/1978).

In 1985, a 10-12% loss in an estimated 2 million chicken per month production rate due to ND was reported in Durban / Natal (The Citizen: 23/10/1985).

A pigeon paramyxovirus was reported in South Africa in the Western province. The outbreak started in 1984 in the Western province, and spread to other regions (The Argus: 18/03/1987). Submission of diseased pigeons to the Poultry section of the Veterinary Research Institute, Onderstepoort, led to the identification of the causative agent as NDV. The virus was classified as a lentogenic strain belonging to PMV-1 serogroup as described by Alexander *et al.*, 1985 (Pienaar & Cilliers, 1987).

A viral epidemic affecting the broiler industry was reported at the beginning of 1990. The causative agent was not stated (Finansies & Tegniek: 23/11/1990). In 1994 a countrywide enzootic of NDV was reported in the majority of the provinces in South

Africa. In Kwazulu-Natal the following areas were affected; Durban, Pietermaritzburg, and the Midlands. Thousands of mortalities were reported in the commercial industry with losses of up to 50-60% (The Star: 15/06/1994). The outbreak started in February and continued until mid April. Dr. Roger Horner (Pathologist fo the Department of Veterinary Services, KZN) states that although NDV is known to be endemic to Natal it had largely been confined to the "kraal chickens". This particular strain however is described as very virulent and managed to break through inoculation programs in commercial flocks (The Star: 1994). The regions largest poultry producer, Rainbow Chicken (RSA), reported high mortalities (The Natal Witness: 27/07/1994). Rainbow Chicken lost an estimated R17.9 million in production for the period March-September (The Star:4/11/1994). This outbreak was said to be the worst in 25 years. The majority of the producers affected were outside the Western Cape province (Business Day: 4/11/1994)

In the Eastern Cape province (RSA) a total mortality rate of 90%was reported. A farmer in Berlin is said to have lost half a million birds over a three week period at a rate of 32 000 fowls per day. A chicken processing factory in Mdantsane, and the following towns in the region were affected King Williams Town, Grahamstown, and many surrounding rural villages. The virus was described as being virulent, leading to death 3-4 days after infection (Eastern Cape Herald: 25/08/1994). Dr Carnie Pienaar (Spokesperson for the Directorate of Agriculture, Eastern Cape) reports that the disease started in the Transvaal (Now Gauteng province), and subsequently spread to the Eastern Cape and other regions in South Africa. A total of three million chickens died during this outbreak, leading to a shortage of poultry and eggs with an accompanying increase in prices (Eastern Cape Herald : 13/09/1994).

Outbreaks were also reported in the Ndanga district (The Southern Province of Masvingo) in Zimbabwe. It was the second NDV outbreak to hit Zimbabwe in that year. It was feared that it would hamper the country's multimillion-dollar poultry and ostrich industry. The country exports ostrich meat and live birds to Europe and the US. Its

poultry products to the value of ten million Rand are also exported to Botswana, Zambia, and Mozambique (The Star:10/06/1994).

In 1998 NDV outbreaks in South Africa were reported in the North-West province in Kuruman and Kanana. Other possible areas affected included Lichtenburg, Gamotlala, Coligny, Ventersdorp and Kudumane (The Star:14/08/1998; The Citizen: 14/08/1998). Outbreaks were also reported in the Mpumalanga, Gauteng, and Northwest Province, affecting specific farms in Lanseria, Standerton and Zeerust. No mortality rates were stated. The outbreak was said to be localized to the "agterplaashoenders", and did not affect commercial poultry (Finansies & Tegniek: 28/08/1998). In contrast the outbreak was described as serious in Gauteng and Mpumalanga provinces according to the newspaper Die Beeld. A high incidence of the disease was reported a few weeks earlier in four poultry farms nearby Lanseria (Die Beeld: 04/08/1998).

Epidemiologically grouped SA strains using RT-PCR technology

The previously mentioned RT-PCR detection method was used to screen 34 NDV isolates from South Africa and Mozambique involved in epizootics between 1990-1995. The results indicated that two aetiological agents were responsible for the outbreak. One genotype VIIb, included isolates from Bulgaria and Turkey, and was traced to Southern Europe (Herczeg *et al.*, 1999). A novel group termed VIII, was found to be unique to South Africa, and is thought to have been maintained in the area by endemic infection (Herczeg *et al.*, 1999).

2.9 Conclusions

Restriction site analysis allows the unambiguous identification of individual strains, this is most important in areas where live vaccines are used (Ballagi-Pordány *et al.*, 1996). It has the advantage over mAb in that it allows grouping of isolates with identical restriction site patterns, but different epidemiological background into molecular subtypes (Lomniczi *et al.*, 1998). It also confirmed differences shown with mAb (Ballagi-Pordány *et al.*, 1996).

When coupled to genetic analysis, this technique reveals the nature of epidemiological relationships between epizootics in geographically widely separated areas (Herczeg *et al.*, 1999). Recently the BESS-T^{TM} Base Reader Kit has been developed for the molecular epidemiological typing of St. Louis Encephalitis Virus (Charrel & Chandler, 1999). It involves PCR amplification with one labeled primer in the presence of limiting amounts of dUTP. The resulting uracil-containing product is enzymatically cleaved at sites of deoxyuridine incorporation resulting in a set of nested fragments that are separated on a sequencing gel (Charrel & Chandler, 1999). It is a relatively low-cost and simple application that can be used to process large numbers of samples (Charrel & Chandler, 1999). It however requires analysis based on computer programs such as multiple alignment procedures and the determination of phylogenetic parameters (Charrel and Chandler, 1999).

The RT-PCR method of Ballagi-Pordány *et al.*, (1996) has been used to generate a tremendous amount of epidemiological information of over 200 NDV strains from nearly all parts of the globe. Using this technique these researchers were able to determine the aetiological agents responsible for outbreaks of ND in South Africa into genotypes VIIb and VIII. This detection method will be used in this study to determine the genotype of NDV isolates from South Africa that were isolated during the outbreak that occurred in the region in 1998, with the aim of determining the genotypes to which they belong

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CHAPTER 3:

THE COLLECTION, AMPLIFICATION AND IDENTIFICATION OF VIRUS ISOLATES

3.1 INTRODUCTION

The investigation of any virus has to begin with finding the best way to cultivate it (Brock & Madigan, 1991). Viruses can only be grown in living matter. This makes the laboratory cultivation of animal viruses more difficult, time consuming, and specialized than the cultivation of other microbial pathogens (Brock & Madigan, 1991).

Four main systems can be used for the cultivation of animal viruses, which are cell cultures, animal experiments, organ cultures, and chick embryos (Primrose & Dimmock, 1998). After critically evaluating these methods, the chick embryo (CE) technique was selected as the best route for viral propagation. This technique was the standard host for the propagation of many human and animal viruses (Primrose & Dimmock, 1998) prior the 1950's (Burleson *et al.*, 1992). The skill required for the inoculation of chicken embryos can be learned in a reasonable time, and it can be performed in primitive laboratories (Primrose & Dimmock, 1998). CE techniques also have the added advantage of producing relatively high virus titres when compared to the other propagation methods mentioned (Primrose & Dimmock, 1998).

Chick embryo (CE) techniques have the limitations of being somewhat space and cost intensive. This technique is, however, the method of choice for the propagation of paramyxoviruses and influenza virus (Burleson *et. al.*, 1992).

Microbial haemagglutination occurs when erythrocytes of a variety of species are agglutinated through the action of microorganisms or their products (Mascoli & Burrell, 1965). The ND virion posseses two glycoproteins spikes associated with the outer side of the lipid envelope (Klenk *et al.*, 1970). The haemagglutinin neuraminidase (HN) glycoprotein, has haemagglutinating and neuraminidase activity (Shimizu *et al.*, 1974).

The HN facilitates agglutination when avian red blood cells are mixed with the viral suspension (Mascoli & Burrell, 1965).

Haemagglutination can, however, be inhibited when viral neutralizing serum is added, a phenomenon known as haemagglutination inhibition (Mascoli & Burrell, 1965), thus, allowing the detection of a particular virus using specific viral antiserum (Mascoli & Burrell, 1965). Haemagglutination inhibition using NDV specific serum, will be used to confirm the presence NDV in allantoic fluid collected after viral propagation in CE.

3.2 REAGENTS AND CHEMICALS

The reagents and chemicals used were of molecular biological or analytical grade. The chemicals used were obtained from the following companies:

Merck, Germany: Na_2HPO_4, KH_2PO_4, $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, $NaHCO_3$, gelatin, barbitone sodium; **Saarchem (Pty) Ltd**: NaCl, glucose, **NT, SA**: citric acid, KCl, **Oxoid, UK**: Tryptose, **Biolab, Merck**: Nutrient broth, bacteriological agar, **Univar, SA**: Sodium citrate, tri-sodium citrate, **AnalaR (BDH), UK**: Diethyl barbituric acid.

3.2.1 History of field strains

The following institutions provided NDV field strains collected during an outbreak of the disease in South Africa in 1998: (Table 3.1).

1. Onderstepoort Veterinary Institute, Agricultural Research Council (Dr. D. Verwoerd).
2. Poultry Reference Laboratory, Faculty of Veterinary Science, Onderstepoort, University of Pretoria.
3. EarlyBird Farms (Standerton).

Table 3.1 **NDV isolates collected from poultry institutions in South Africa. The original accession numbers as supplied by the various institutions are indicated.**

Strain/ Accession number	Source	Isolation location (city)
Onderstepoort Veterinary Institute, ARC (Dr. Dirk Verwoerd) [a]		
1. M269/98	(V org. pool)	Koppies
2. M28/98	(Av. Pool 1AS)	Randfontein
3. M229/98	(Ostr. Pool)	Calitz dorp
4. M89/98	(Ostr. Pool 1)	Rustenberg
5. M89/98	(Av pool 2)	Rustenberg
6. M281/98	(Av pool)	Kroonstad
7. M308/98	(Av pool)	Sinoville
8. M183/98	(Ost. Liv. B)	Oudtshoorn
9. M193/98	(Ost pool 5)	Oudtshoorn
10. M219/98	(Av. trachea 2 AS 10^{-5})	Kroonstad
11. M56/98	(Av. Pool 1AS)	Seymour
12. M57/98	(Av pool 1 1AS)	Graaff Reniet
13. M193/98	(Ost. Pool 6)	Oudtshoorn
University of Pretoria (UP) Poultry Reference Laboratorium [b]		
	Original identification	
14. 1109/98	(NDV) broilers, (trachea)	Boons
15. 1112/98	(NDV) broilers, (trachea)	Lebowakgomo
16. 1159/98	(NDV) layers, (brain, kidney)	Ventersdorp
17. 1163/98	(NDV) Unknown	Unknown
18. 1193/98	(Paramyxovirus) Broilers, (trachea, brain)	Ermelo
19. 1309/98	(Paramyxovirus) Parrots (trachea, lung)	Quarantine
20. 1376/98	(Paramyxovirus) Broilers (trachea)	Nigel
Early Bird Farms [c]		
21. 725/98	(NDV)	Standerton
22. 719/98	(NDV)	Standerton

[a]Strains from the Onderstepoort Veterinary Institute and UP Reference Laboratory indicate the avian source from which the isolates were isolated initially. [b]Field strains from UP Reference laboratory and EarlyBird Farms include their original classification as either NDV or paramyxoviruses.

3.3 AMPLIFICATION OF VIRAL STRAINS IN CHICKEN EMBRYOS

Reconstitution of Freeze-Dried field strains and Reference strain LaSota

Freeze-dried samples were resuspended in 1ml phosphate buffered saline (PBS) pH 7.4 (0.14M NaCl, 0.0027M KCl, 0.010M Na_2HPO_4, 0.0017M KH_2PO_4), or Tryptose phosphate broth (TPB) (20.0g/l tryptose, 0.086M NaCl, 0.018M Na_2HPO_4, 0.010M glucose) supplemented with Ampicillin (Boehringer Mannheim, Germany) to a final concentration of 50µg/ml. (Kant *et al.*, 1997; Ausubel *et al.*, 1997) and filter sterilized through a 0.22µm filter (Osmonics). The filtrate was incubated at room temperature for approximately 2 hours. The reference NDV LaSota strain was reconstituted in 5ml RNase free H_2O as described by the manufacturer (Lentogen LaSota, Live attenuated freeze-dried vaccine against ND, isbis p.a. Biopharmaceutical Research and Production Laboratories Chingnolo Po-Parvia, Italy).

Amplification in chicken embryos

Isolates were passaged in white, SPF leghorn eggs obtained from (Glen Agricultural College, Poultry Division, Bloemfontein) and pre-incubated for 9-10 days. After demarcation of the air sac, and disinfecting the shell area around the inoculation site with a 1% (v/v) Virukill (ImmunoVet Services, SA) solution, a hole was punched at the appropriate site. Aliquots of 100µl of either allantoic fluid, or of each of the viral filtrates of the freeze-dried samples were inoculated into the allantoic cavity. This was done in triplicate for each sample. The opening in the eggs were sealed with wood glue (Ponal, Henkel SA (Pty) (ltd.), incubated at 37°C and candled at 24hour intervals. Candling was performed by exposing the eggs to the light beam produced by the light source of a stereomicroscope. Eggs which showed embryo death after 24 hours, indicated by decreased embryo movement, and a collapsed vascular system were discarded, as the cause of death at this early stage could be attributed to mechanical shock or bacterial contamination (Versteeg, 1985; Mascoli & Burrell, 1965).

Harvesting of the allantoic fluid.

The eggs were chilled overnight once embryo death was observed, or after they had been incubated for 72 hours. The shell area around the inoculation site was disinfected with a

1%(v/v) Virukill solution and aseptically removed. The allantoic fluid was collected in sterile McCartney bottles and aliquots thereof were streaked onto nutrient agar plates (NA) (16g/l nutrient broth, 20g/l bacteriological agar)/BTA (33g Tryptose blood agar base, sheep blood to 10% of the medium) to check for bacterial contamination. The collected allantoic fluids were stored at -70°C (Specht Scientific Instruments, S.A.) until further use.

3.4 HAEMAGGLUTINATION (HA) AND HAEMAGGLUTINATION INHIBITION TESTS (HI)

Preparation of a 0.3% Red Blood Cell (RBC) concentrate:

Chicken red blood cells (RBC) were collected in a 1:1 ratio with Alsevers solution (0.10M glucose, 0.072M NaCl, 0.027M tri sodium citrate, 0.0026M citric acid) and pelleted by centrifuging at 3 X 1000g for 5 minutes (Beckman model J2-J21, Beckman Instruments, UK). The supernatant was discarded and the cells re-suspended in the desired volume of Veronal buffer (9mM barbitone sodium, 0.024mM $MgCl_2.6H_2O$, 0.51mM $CaCl_2.2H_2O$, 15mM $NaHCO_3$) and centrifuged as above. This washing process was repeated at least three times, or until a clear supernatant was observed. Concurrently a 0.1% gelatin solution in 100ml Veronal buffer was prepared. A 0.3ml volume of the packed red blood cell pellet was added to the Veronal buffer-gelatin solution after the last centrifugation step to give a final concentration of a 0.3% RBC.

Preparation of Virus concentrate

Allantoic fluid samples were clarified by centrifuging at 5 X 1000g for 10 minutes.

Haemagglutination Test

Serial two-fold dilutions of the allantoic fluid were performed in V-bottomed wells of a plastic, 96 well microtiter plate (Nunc, Denmark) containing 50µl Veronal buffer. This was achieved by mixing 50µL of allantoic fluid with 50 µL of Veronal buffer in the first well of each row. A 50µL aliquot of the mixture in the first well was transferred to the following well and the process repeated for all subsequent wells in the row. The last

50µL aliquot was discarded after mixing. No allantoic fluid was added to the red blood cell control wells.

Agglutination was allowed to proceed for 60 minutes at room temperature after the addition of 50µl of the 0.3% RBC concentrate, and agglutination read at 100% agglutination. 100% Agglutination was read as the dilution at which no observable RBC pellet was observed at the bottom of the wells. The HA titre was read as the last dilution of virus that caused complete agglutination (Bragg, 1995) and is expressed as the number of haemagglutinating units (HA units) per unit volume (Burleson *et al.*, 1992). To perform haemagglutination inhibition tests, allantoic fluid used in the HA test had to be diluted to four HA units (the diluted antigen control). This was achieved by diluting the concentrated allantoic fluid, to the dilution observed in a well located two rows prior to that in which 1HA unit was read. The positive control reaction was performed using allantoic fluid obtained from the passage of the commercially available NDV vaccine strain LaSota.

Haemagglutination Inhibition

Two fold serial dilutions were performed in respective wells of a V-bottomed microtitre plate containing 50µl Veronal buffer, using 50µl positive serum (SPAFAS, S.A.), negative serum (obtained from SPF chicken, Poultry Unit, Glen Agricultural School), 4HA (HA diluted) respectively. To each of the wells to be tested, 50µl of a virus solution containing 4 HA units was added except the RBC and antigen (Ag) control wells. After allowing the reaction to proceed at constant room temperature for 30 minutes, 50µl of the 0.3% RBC concentrate was added to each well. After incubation at a constant room temperature for 60 minutes, the results were read at 50% end point (Burleson *et al.*, 1992). End point was defined as the last dilution of virus showing complete agglutination and containing one HA unit (Burleson *et al.*, 1992)

3.5 RESULTS AND DISCUSSION

3.5.1 Embryo mortality in chicken eggs and HA/HI tests

Table 3.2 Average embryo mortality. END (embryo not dead): Indicates cases in which embryo death was not observed after 5-6 days post inoculation.

Dr. Dirk Verwoerd (Ondertepoort Veterinary Institute, University of Pretoria)		
Strain/ Accession number	**Source**	**Average embryo mortality (hr/days)**
1. M269/98	(V org. pool)	80hrs/ ~3 days
2. M280/98	(Av. Pool 1AS)	END
3. M229/98	(Ostr. Pool)	END
4. M89/98	(Ostr. Pool 1)	48hrs/ ~2 days
5. M89/98	(Av pool 2)	96hrs/ ~4 days
6. M281/98	(Av pool)	END
7. M308/98	(Av pool)	END
8. M183/98	(Ost. Liv. B)	64hrs/ ~3 days
9. M193/98	(Ost pool 5)	72hrs/ ~3 days
10. M219/98	(Av. trachea 2 AS 10^{-5})	72hrs/~3 days
11. M56/98	(Av. Pool 1AS)	80hrs/ ~3 days
12. M57/98	(Av pool 1 1AS)	48hrs/ ~2 days
13. M193/98	(Ost. Pool 6)	40hrs/ ~3 days
University of Pretoria (UP) Poultry Reference Laboratory		
	Original identification	
14. 1109/98	(NDV)	72hrs/ ~3 days
15. 1112/98	(NDV)	72hrs/ ~3 days
16. 1159/98	(NDV)	72hrs/ ~3 days
17. 1163/98	(NDV)	72hrs/ ~3 days
18. 1193/98	(Paramyxovirus)	72hrs/ ~3 days
19. 1309/98	(Paramyxovirus)	144hrs/ ~3 days
20. 1376/98	(Paramyxovirus)	72hrs/ ~3 days
Early Bird Farms		
21. 725/98	(NDV)	END
22. 719/98	(NDV)	END

Table 3.3 HA and HI test results obtained using allantoic fluid harvested after viral field strain passage in embryonated eggs. ND, in this context indicates that HA/HI tests were not performed as initial RT-PCR detection gave positive results. **Key:** + In the case of HA tests, indicates agglutination, – indicates no agglutination. In the HI tests +, denotes haemagglutination inhibition, – indicates no inhibition of agglutination

Dr. Dirk Verwoerd (Ondertepoort Veterinary Institute, University of Pretoria)			
Strain/ Accession number	**Source**	**Haemagglutination Test/ (HA)**	**Haemagglutination Inhibition Test/ (HI)**
1. M269/98	(V org. pool)	–	–
2. M280/98	(Av. Pool 1AS)	–	–
3. M229/98	(Ostr. Pool)	–	–
4. M89/98	(Ostr. Pool 1)	+	+
5. M89/98	(Av pool 2)	–	ND
6. M281/98	(Av pool)	–	–
7. M308/98	(Av pool)	ND	ND
8. M183/98	(Ost. Liv. B)	+	–
9. M193/98	(Ost pool 5)	+	–
10. 19	(Av trachea 2 AS 10^{-5})	–	–
11. M56/98	(Av. Pool 1AS)	–	–
12. M57/98	(Av pool 1 1AS)	ND	ND
13. M193/98	(Ost. Pool 6)	+	–
University of Pretoria (UP) Poultry Reference Laboratory			
	Original identification		
14. 1109/98	(NDV)	–	–
15. 1112/98	(NDV)	–	–
16. 1159/98	(NDV)	–	–
17. 1163/98	(NDV)		–
18. 1193/98	(Paramyxovirus)	–	–
19. 1309/98	(Paramyxovirus)	–	–
20. 1376/98	(Paramyxovirus)	–	–
Early Bird Farms			
21. 725/98	(NDV)	–	–
22. 719/98	(NDV)	–	–

Of the 22 strains amplified, approximately 60% had an average embryo mortality of 3 days. Strains M89/98 (Ost Pool 1) and M57/98 caused embryo death in approximately 2 days and no embryo death was observed with 6 of the field strains (Table 3.5.1)

Strain M89/98 (Ost Pool 1) was used as a positive control field strain in HA/HI tests. It was one of the field strains positively identified as NDV upon the first attempt at RT-PCR. It was used to find a correlation between HA/HI detection and RT-PCR detection. In order to determine the specificity of both these tests.

In this study, the molecular epidemiology of NDV strains isolated during an epizootic of Newcastle disease in South Africa in 1998 was investigated. Initially it was assumed that the isolates received from various poultry institutions were NDV (Table 3.1). The majority of the samples obtained were clearly identified as either NDV or paramyxoviruses by the supplying institution.

Upon arrival, the isolates were passaged in 9-to-10 day-old SPF chicken embryos. This was done in order to obtain larger volumes of virus for future work. During the incubation period, embryo mortality was recorded to serve as an indication of virulence.

Conventionally the virulence of NDV field strains is determined by performing a series of tests including the Mean Death Time (MDT), Intracerebral Pathogenicity Index (ICPI), and the Intravenous Pathogenicity Index (IVPI) test (Alexander, 1991). These tests were not performed in this study. The Mean Death Time Test is performed by making a series of 10-fold dilutions of the virus in sterile isotonic saline, and 100μL of each dilution inoculated into the allantoic cavity of at least five 9-to-10 day-old embryonated eggs. Another five eggs are inoculated eight hours after the first inoculation, at each dilution. The eggs are incubated at 37°C for seven days and the times at which the embryos are first observed as being dead are recorded. The MDT is the average time in hours for the minimum lethal dose (the highest dilution at which all the eggs die) to kill the embryos (Alexander, 1991). The following MDT are suggested for determining virulence of NDV

strains, <60hrs for velogenic strains, 60-90hrs for mesogenic strains, >90hrs for lentogenic strains (Alexander, 1991).

Two strains appeared to be lentogenic when considering embryo mortality only. These strains are 1309/98 and M89/98 (Av Pool 2) that displayed embryo mortality greater than 96 hours. Strain 1309/98, however gave negative results with RT-PCR amplification (Chapter 4). Positive RT-PCR results were obtained with strain M89/98 (Av Pool 2) positively identifying it as NDV. The fusion protein cleavage site of this strain also posses the characteristic amino acid sequence observed in lentogenic strains (Chapter 5).

Virus filtrates of reconstituted freeze-dried samples, or aliquots of allantoic fluid of each of the field strains were inoculated into three 9-to-10 day-old embryonated SPF eggs. The embryo mortality was calculated as the mean time in hours that embryo death was observed for each particular virus strain. Upon comparison of embryo mortality of the field strains to the MDT values indicative of the different levels of virulence, strains M89/98 (Ost Pool 1) and M57/98 (Av Pool 1 AS) with embryo mortality of 48 hours were observed to be velogenic. Preliminarily, based only on embryo mortality, one could suggest that the majority of the strains were mesogenic, causing embryo death between 60-90 hours post infection (Table 3.2).

A number of strains did not cause embryo death within the amplification period, suggesting that they were lentogenic strains. However, upon performing RT-PCR tests no amplification products were obtained with the majority of the strains (Chapter 5). Of the twenty-two field strains obtained, four strains gave positive RT-PCR results. These include those that displayed embryo mortality suggestive of mesogenic strains, such as strains M269/98 (V. Org Pool) with embryo mortality of 80 hours, and M183/98 (Ost. Liv. B) with embryo mortality of 64 hours.

Strain M193/98 (Ost Pool 6) caused embryo death in 40 hours suggesting that it could be a velogenic NDV strain. However, no amplification product was obtained when genomic RNA extracted from this strain was subjected to RT-PCR. Because the majority of the

strains gave negative results upon RT-PCR amplification, this prompted an investigation into the initial assumption that the strains received were NDV. RT-PCR failure can be attributed to various factors, including the observation that the viruses obtained may not have been NDV as initially assumed. It was therefore decided to perform HA/HI tests to determine whether the strains that did not produce amplicons upon RT-PCR where NDV.

Strain M89/98 (Ost Pool 1), gave positive RT-PCR results, and was used as a positive control strain in the HA/HI tests. Strains M89/98 (Av Pool 2), M308/98 (Av Pool), and M57/98 (Av Pool 1 1AS) also gave positive RT-PCR results and were therefore not subjected to HA/HI tests.

Negative results were obtained for both HA and HI tests with the majority of the strains tested (Table 3.3). This group included strains with embryo mortality that suggested the presence of mesogenic NDV isolates. Embryo death observed within this group suggests the presence of a viral pathogen, and the HA/HI test results indicate that this pathogen is not NDV, as haemagglutination was not observed. Lack of haemagglutination, however, does not mean that a pathogen is not present, as not all viruses can agglutinate RBCs. In addition conditions under which haemagglutination occurs, vary from virus to virus. Some only agglutinate RBCs of certain animal species, others agglutinate only under carefully defined pH, whilst others require a saline environment. Incubation temperature may also play an important role in haemagglutination tests (Burleson *et al.*, 1992).

In addition to the positive control strain M89/98 (Ostr Pool 1), three other field strains agglutinated a 0.3% preparation of chicken erythrocytes, namely M183/98 (Ost Liv B), M193/98 (Ost Pool 5), and M193.98 (Ost Pool 6). Haemagglutination occurs due to a direct interaction between the haemagglutinin located on the viral surface, and the red blood cell (Burleson *et al.*, 1992). These strains agglutinated chicken erythrocytes, and displayed embryo mortality indicative of the presence of pathogenic agents. However, no loss in haemagglutinating activity was observed upon incubation of the above mentioned strains with anti-NDV serum. The quality of serum used could be a factor contributing to the lack of inhibition. However, inhibition was observed with the positive control strain

M89/98 (Ost Pool 1), and the vaccine strain LaSota (results not shown), using the same serum. This leads us to conclude that field strains M183/98 (Ost Liv. B), M193/98 (Ost Pool 5), and M193/98 (Ost Pool 6) are not NDV. These strains also failed to be amplified by RT-PCR, using primers specific for NDV (Chapter 5).

It can be speculated that the above mentioned strains could belong to other genera or species within other viral families capable of agglutinating red blood cells, such as adenoviridae, bunyaviridae, coronaviridae, flaviviridae, orthomyxoviridae, paramyxoviridae, coronaviridae, parvoviridae, picornaviridae, poxviridae, reoviridae, rhabdoviridae, and togaviridae (Burleson *et al.*, 1992). Specifically these viruses could belong to viral families known to be chicken pathogens, capable of agglutinating chicken red blood cells.

It is thought that these strains may be avian influenza virus, as these isolates were collected from ostrich hosts, from which avian influenza has been previously isolated in South Africa (Bragg, personal communication).

Strain M308/98 (Av Pool) did not cause embryo death, but grouped along with velogenic strains in genotype VIIb (Chapter 5). In addition this strain possessed at its fusion protein cleavage site an amino acid sequence characteristic of mesogenic, or velogenic strains. In this case embryo mortality observed failed to correctly identify the virulence of this strain. This could have resulted due to a variety of factors. It should be considered that the sample may not have contained any infectious particles upon passage in allantoic fluid. It however, may have contained intact nucleic acid that allowed positive RT-PCR amplification.

Bacterial contamination was found in certain cases (data not shown), despite reports that chick embryo techniques can be performed in primitive laboratories as the embryo provides a sterile environment for viral growth. The source of the contamination might have been airborne or acquired at the point of harvesting allantoic fluid, as contamination

occurred in a haphazard manner. This problem was eliminated via antibiotic treatment and filter sterilization.

Future research should entail a thorough investigation into the identity of the unknown pathogens. This could be achieved by performing virus neutralization studies, or performing PCRs with primers specific for other chicken pathogens.

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CHAPTER 4:

OPTIMISATION OF RNA EXTRACTION AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) FOR NDV

4.1 INTRODUCTION

NDV contains a single stranded RNA molecule as its genomic materials. To facilitate the amplification of regions on the genome, the RNA has to be extracted and amplification performed by means of RT-PCR. Genomic RNA was extracted from NDV virions passaged via the allantoic route in chicken embryos. Numerous techniques exist for the isolation of RNA from microorganisms. The choice of an RNA isolation method is dependent upon the type of RNA to be purified, and its relative abundance. The RNA source, sample size and the relative convenience of the isolation procedure, are also factors that should be considered (Promega, Protocols and Applications Guide).

The amplification of NDV in embryonated eggs via inoculation into the allantoic fluid releases intact virions into this medium. The allantoic fluid, the fluid content of the chorioallantoic sac, contains waste products and ureates produced by the developing embryo (Burleson *et al.*, 1992). Allantoic fluid increases in size as the embryo develops, producing large sample volumes of virus containing fluid (Burleson *et al.*, 1992), from which genomic RNA can be extracted.

The NDV genome is a negative-sense, single stranded non- segmented RNA molecule (Phillips *et al.*, 1998). The RNA genome consists of approximately 15 000 nucleotides (de Leeuw & Peeters, 1999), that encode six genes (Millar & Emmerson, 1982).

The NDV RNA genome exists as a helical ribonucleoprotein (nucleocapsid), surrounded by a host derived plasma membrane or lipid bilayer (Klenk *et al.*, 1977; Landberger *et*

al., 1971; Klenk & Choppin, 1969). These protective structures, the nucleocapsid protein, and the lipid bilayer are targets for many RNA extraction procedures (Promega, Protocols and Applications Guide). The specific use of guanidine thiocyanate and N-lauroyl sarcosine in RNA extraction procedures is aimed at disrupting nucleoprotein complexes allowing the release of protein free intact RNA into solution (Promega, Protocols and Applications Guide)

The effective use of RNA in procedures such as RT-PCR, Northern blotting, cDNA synthesis and *in vitro* translation is greatly affected by the purity and integrity of isolated RNA (Promega, Protocols and Applications Guide). Four important requirements must be met for the successful isolation of intact RNA: 1) effective disruption of the cell or tissues; 2) denaturation of nucleoprotein complexes; 3) endogenous ribonuclease (RNase) inactivation; and 4) purification of RNA from contaminating DNA and protein (Promega, Protocols and Applications Guide).

RNA isolation is synonymous with inhibiting RNase activity. During RNA extraction and storage, the action of RNases must be inhibited, as RNA is highly susceptible to degradation by these enzymes (Sambrook *et al.*, 1989). In contrast to deoxyribonucleases (DNases), RNases are difficult to inactivate, as they do not require co-factors, are heat stable, and refold following heat denaturation (Promega, Protocols and Applications Guide). RNase activity can be inhibited by the use of known RNase inhibitors, guanidine thiocyanate or β-mercaptoethanol (Chirgwin *et al.*, 1979). Performing these procedures on ice can also slow the rate of RNA degradation (Han *et al.*, 1987).

Following genomic RNA extraction, from the field strains obtained, specific regions of the RNA template were amplified for further analysis using RT-PCR. The polymerase chain reaction (PCR) can be described as an *in vitro* technique for the amplification of DNA or cDNA template (Saiki *et al.*, 1985). PCR has replaced cloning as the DNA amplification method of choice (Arnheim & Erlich, 1992). PCR has the advantages of generating sufficient material for subsequent analysis; it is performed *in vitro*, is sensitive and requires relatively little starting material. It is a simple technique that produces

results in a relatively short period of time when compared to the time required for cloning (Arnheim & Erlich, 1992).

Thermostable polymerases (DNA dependent DNA polymerases) used in PCR require a DNA template (Saiki *et al.*, 1985). In order to study the differential expression of genes in tissues during development, or amplify regions of viral RNA genomes the RNA template has to be reverse transcribed to cDNA to provide the DNA template for the thermostable polymerase (Sambrook *et al.*, 1989). This is achieved through a process known as reverse transcription, hence RT-PCR.

The initial step in RT-PCR involves the use of Avian Myeloblastosis virus (AMV), or Moloney murine leukemia virus (M-MLV or MuLV) reverse transcriptase enzymes (RNA dependent DNA polymerases) to reverse transcribe the RNA template. Polymerisation extends from the hydroxyl end of random primers, or sequence specific primers (Promega, 1996) complementary to particular sequences on the RNA template. Polymerisation will extend until a DNA sequence complementary in base sequence to the sequence flanked by the primers is obtained.

After the initial reverse transcription step, the procedure follows the temperature cycling steps of basic PCR, amplifying the target sequence as a DNA sequence.

The essential reaction components of a PCR reaction mixture include, template DNA (or cDNA), a thermostable DNA polymerase, two oligonucleotide primers, deoxyribonucleotide triphosphates (dNTPs), reaction buffer and magnesium (Arnheim & Erlich, 1992). Subsequently, the reaction components are mixed and placed in a thermal cycler, set for a predetermined number of cycles. Each amplification cycle doubles the amount of target DNA sequence in the reaction by subjecting it to a temperature series for varying lengths of time. The temperature series is initiated by a denaturation step at approximately 95°C for between 15 seconds to two minutes. It separates the DNA helix to form single stranded DNA molecules, that act as template for thermostable polymerases (Promega, 1996).

Subsequent to template DNA denaturation, the temperature is reduced to approximately 40-60°C for between 30-60 seconds to allow the oligonucleotide primers to form a stable association with the separated DNA strands. Chain elongation is initiated by an increase in temperature to approximately 74°C for a period of 1-2 minutes. This is the optimum reaction temperature for thermostable polymerases (Arnheim & Erlich, 1992). At this temperature the polymerase catalyses a tail growth mechanism, whereby complementary nucleotide bases are added to the hydroxyl end of the primer, synthesizing a copy of each strand of the target template (Promega, 1996). The end of the elongation step signifies the completion of one cycle of amplification.

PCR mimics DNA dependent DNA synthesis during chromosomal replication, or DNA repair in cells (Promega, 1996). In contrast to cellular conditions, PCR cycling conditions and reaction components are not ideal, and have to be optimized for each template primer pair combination. Factors affecting PCR, such as the quantity of template, the enzyme of choice, primer design and the amplification conditions used are important for successful amplification results (Grunenwald, 1999).

The versatility, speed, and sensitivity of PCR has led to its application in many areas in biology, such as immunology, forensic science, evolutionary biology, and ecology (Arnheim & Erlich, 1992). In this study RT-PCR was used to amplify the following sequences of the NDV viral genome:

1. F gene nt 334-1682 (1349bp product)
2. M gene nt 44-1141 (1098bp product)
3. M gene nt 1163-F gene nt 492 (557bp product)

The amplification products of the M and F genes served as substrates for restriction enzyme (RE) analysis and the intergenic amplimer was sequenced (Chapter 5).

As mentioned above the isolation of intact RNA is crucial for its effective use in a procedure such as RT-PCR. In addition, the amplification of specific regions using RNA as starting template is very precarious. In this study, various methods of RNA isolation,

as well as different amplification procedures using RT-PCR were tested. In addition the RT-PCR protocol for each primer pair had to be optimised for efficient amplification.

4.2 REAGENTS AND CHEMICALS

The reagents and chemicals used were of molecular biological or analytical grade. The chemicals used were obtained from the following companies:
Merck, Germany: EDTA, chloroform, isopropanol, EDTA, SDS; chloroform, **Saarchem (Pty) Ltd:** NaCl, **Univar, SA**: tri-sodium citrate, **Boehringer-Mannheim, Germany**: Tris base, Taq Polymerse, dNTP mix, Herxanucleotide mix, cDNA synthesis Kit, proteinase K, **Univar, S.A.**: absolute ethanol, **Sigma, USA:** N-Lauroylsarcosine sodium salt, isoamylalcohol LiCl, **Macherey-Nagel, Germany**: NucleoSpin Extract 2 in 1 Kit, **Gibco-BRL:** Trizol/Tri reagent, **USA, Promega, USA.**: RNasin, Moloney Murine Leukimia Virus Reverse Transcriptase (M-MLV), Access RT-PCR Kit, **Integrated DNA Technologies Inc, Whitehead, South Africa:** Oligonucleotide Primers, **BioSolve (ltd.), Netherlands**: biophenol, **Promega, USA.**: nuclease free ddH_2O.

4.3 RNA EXTRACTION

RNA extraction method 1

RNA was isolated from 50µl volumes of clarified allantoic fluid as described Sambrook *et al.* (1989), for the isolation of Total RNA from eggs and embryos.

Approximately 10 volumes of homogenization buffer (50mM NaCl, 50mM Tris·Cl (pH 7.5), 5mM EDTA (pH 8.0), 0.5% SDS, 20µg/ml proteinase K) were thoroughly mixed with 50µl infective allantoic fluid. The homogenate was incubated for an hour at room temperature, with occasional mixing. RNA was extracted into the aqueous phase by the addition of an equal volume of biophenol and separated (5 X 1000g for 10 minutes at room temperature) (Eppendorf centrifuge 5417R, Germany). The aqueous phase was transferred to a new tube and re-extracted as stated above. Finally, the nucleic acid was precipitated (–20°C for at least 2 hours) by the addition of 0.1volumes of 3M sodium

acetate (pH 5.2), and 2.5 volumes of ice-cold 100% ethanol. The nucleic acid was pelleted (5 X 1000g for 15 minutes at 4°C) and air-dried. The nucleic acid pellet was re-suspended in 5µl RNase free ddH_2O, mixed with an equal volume of 8M LiCl and stored at–20°C for at least 3 hours or overnight for the removal of yolk, glycoproteins and other contaminants found in embryonic tissues.

The RNA was pelleted (10 X 1000g for 30 minutes at 4°C) and washed with 1ml 75% w/v ethanol and the dried under vacuum (Savant, USA). The RNA pellet was resuspended in 10µl RNase free twice distilled (dd) H_2O and 3 volumes 98% ethanol for storage at –70°C (Specht Scientific Instruments, S.A.).

RNA extraction method 2

A modification of the method described above (Sambrook *et al.*, 1989), was used for the extraction of genomic RNA from NDV containing allantoic fluid. The modifications made were based on the RNA extraction method described by Collins *et al.*, (1993). Briefly, the virions were pelleted (13 X 1000g for 30 minutes) and the pellet resuspended in 1ml TNE buffer (10mM Tris·Cl (pH7.4), 10mM NaCl, 1mM Na_2·EDTA). RNA was extracted by the addition of 10µl SDS 10% (w/v) and incubated at 56°C for 20 minutes. Proteins extraction was facilitated by the addition of phenol: chloroform: isoamylalcohol (25:24:1) and RNA extraction performed according to the method described by Sambrook *et al.*, 1989.

RNA extraction method 3

This method is recommended for the purification of viral nucleic acids from plasma, serum, and cell-free biological fluids. It is based on the use of guanidine thiocyanate containing buffers for RNA extraction, and final elution is accomplished using chromatography.

A 150µl volume of allantoic fluid was mixed with 600µl of RAV1, an RNA-carrier containing buffer, and incubated for 10 minutes at room temperature. Turbid solutions

were clarified (14 X 1000g for 1 minute) and the supernatant mixed with 600µl 98% (v/v) ethanol by vortexing.

The nucleic acid was allowed to bind to a NucleoSpin column placed in a 2ml centrifuge tube by centrifugation (6 X 1000g for 60 seconds at room temperature). Residual lysis solutions were centrifuged as above and the supernatant discarded. A volume of 500µl RAV3 was used to wash the NucleoSpin cup (8 X 1000g for 30 seconds), the flow-through discarded and the washing step repeated. Final removal of RAV3 was accomplished by transferring the NucleoSpin column to a new 2ml centrifuge tube and centrifuging at 14 X 1000g for 5 minutes.

Once the NucleoSpin column was placed in a new sterile 1.5ml eppendorf tube and 50µl RNase free ddH_2O (preheated to 70°C) added, the nucleic acid was eluted by centrifugation (60 seconds at 8 X 1000g).

RNA extraction method 4

In a sterile RNase free 1.5ml eppendorf tube, 200µl allantoic fluid was mixed with 1ml TRIZOL reagent by vortexing. Complete disassociation of the nucleoprotein complex was facilitated by incubation at room temperature for 5 minutes. A 200µl volume of chloroform per 1ml TRIZOL reagent was added to the homogenate and mixed. The solution was vortexed vigorously for 15 seconds. Following incubation at room temperature for 15 minutes, the mixture was centrifuged (12 X 1000g for 15 minutes at 4°C). This allowed the separation of the lower red phenol-chloroform phase, from the interphase, and the colourless RNA containing upper aqueous phase. The aqueous phase was transferred to a new tube and RNA precipitated by mixing with 500µl isopropanol per 1ml TRIZOL reagent used.

Incubation at room temperature for 10 minutes was followed by the collection of RNA (12 X 1000g for 8 minutes at 4°C). After the supernatant was discarded, the RNA pellet was washed by the addition of 1ml 75% ethanol per 1ml TRIZOL reagent initially used, and centrifuged (7.5 X 1000g for 5 minutes at 4°C). The ethanol was removed and the

pellet briefly air-dried. The RNA pellet was resuspended in 50µl sterile RNase free millipore water and incubated at 55-60°C for 15 minutes. RNA preparations were aliquoted into 5µl fractions and stored at -70°C for further use.

In an attempt to increase the concentration of RNA per isolate, RNA pellets of the same isolate were resuspended in a total volume of 50µL nuclease free ddH_2O. In contrast to re-suspending each pellet in 50µL nuclease free H_2O.

4.4 RT-PCR AMPLIFICATION

Following RNA extraction, the following regions of the NDV genome were amplified using RT-PCR:

1. Fusion protein gene, nt 334-1682 (1349bp product) using primers ONDV1aa, and ONDV4aa. The resulting amplicon was subjected to RE analysis using Hinf I, BstO I, and Rsa I.
2. Matrix protein gene, nt 44-1141 (1098 bp product) using primers M1, and M2. The amplicon produced was subjected to RE analysis using enzymes MbO I, and Hinf I.
3. Fusion protein cleavage site amplification was accomplished by means of nested PCR. The first PCR reaction was performed using primes K1, and K2 (M gene nt 778-F gene nt 545). Resulting in a 1008bp product, which was subsequently used as template for nested PCR using primers MV1 and B2 (M gene nt. 1163 - F gene nt. 492)? The final nested PCR product (557bp) was sequenced, and subjected for phylogenetic analysis.

NDV Strains

The following NDV strains were used in positive control reactions, V10, V11, V31, obtained from the Veterinary Biotechnology Group (Department of Microbiology and Biochemistry, UOVS) culture collection and LaSota (Lentogen LaSota, Live attenuated freeze-dried vaccine against ND, isbis p.a. Biopharmaceutical Research and Production Laboratories Chingnolo Po-Parvia. Italy).

4.4.1 FUSION PROTEIN AMPLIFICATION: (F gene nt 334-1682)

One-Step RT-PCR method 1

Genomic RNA extracted from LaSota virions in allantoic fluid using all four RNA extraction methods was used as template for amplification. The oligonucleotide primers used, were published by Herczeg *et al.*, 1999:

ONDV1aa (R): 5'-TGA STC WAT TCG SAR GAS ACA AGR KTC TG-3' (nt 334-362)
ONDV4aa (F): 5'-ATC TGR YCS AGT GTR TTA TTC CCA AGC CA-3' (nt 1654-1682),
(Herczeg *et al.*, 1999)

Reaction volumes of 50µl containing 1 X AMV/Tfl Reaction Buffer, 0.2mM of each of the deoxyribonucleotide triphosphates (dNTPs), 50pmol of each of the primers, 1mM $MgSO_4$, 5U of the enzymes AMV Reverse Transcriptase and Tf1 DNA polymerase were prepared. Reverse transcription was performed at 48°C for 45 minutes, followed by AMV inactivation, and RNA/primer denaturation at 94°C for 2 minutes. Amplification was performed in a thermal cycler (Perkin-Elmer, USA) set for 5 cycles of a 30 second denaturation period at 94°C, a 30 second annealing period at 55°C and a 1 minute 30 second extension time at 72°C. This was followed by 30 cycles of denaturation at 94°C for 30 seconds, and annealing at 48°C for 1 minute, and final extension at 68 for 7 minutes. The cycling parameters were a combination of those recommended by the manufacturer (Promega, Protocols and Applications Guide) and those published by Ballagi-Pordány *et al.*, (1996), specifically for the amplification of the fusion protein gene sequence of interest.

Optimisation of $MgCl_2$ concentration for PCR amplification

Lack of success with method 1, prompted an investigation into the optimization of the reaction components and cycling parameters for amplification. A pUC 18 plasmid, containing the entire LaSota fusion protein gene (Albertyn 1999), was used as template in a series of reactions of varying $MgCl_2$ levels. Oligonucleotides ONDV1aa and ONDV4aa were used as primers (Table 4.1.).

Table 4.1 Optimisation schedule of $MgCl_2$ content in PCR mixtures for amplification of a 75% region of the fusion protein of NDV strain LaSota cloned into the plasmid pUC 18.

Reaction Component	1 (with $MgCl_2$ 15mM)	2 (no $MgCl_2$)	3 (no $MgCl_2$)	4 (no $MgCl_2$)	5 (no $MgCl_2$)
10 X Taq Buffer	2 X	2 X	2 X	2 X	2 X
dNTP's (10mM)	0.2mM	0.2mM	0.2mM	0.2mM	0.2mM
ONDV1aa (100pmol/µl)	2µM	2µM	2µM	2µM	2µM
ONDV4aa (100pmol/µl)	2µM	2µM	2µM	2µM	2µM
pUC 18 + F.P	1µl	1µl	1µl	1µl	1µl
Taq polymerase (5U/µl)	5U	5U	5U	5U	5U
$MgCl_2$ (25mM)	-	0.75mM	1.75mM	3.75mM	4.75mM
d.d.H_2O	30µl	33.5µl	31.5µl	27.5µl	25.5µl
Total Volume	50µl	50µl	50µl	50µl	50µl

The cycling parameters were a modification of those of Ballagi-Pordány *et al.*, 1996, to include a hot start, (denaturation) at 94°C for 5 minutes. Amplification was carried out using 5 cycles of 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 1minute, followed by 30 cycles of 94°C for 45 seconds, 48°C for 1 minute and 72°C for 3 minutes. Finally, extension at 72 °C was performed for 7 minutes (Ballagi-Pordány, *et al.*, 1996).

One-Step RT-PCR method 2

Genomic RNA was isolated from a freeze-dried sample of a known NDV strain V11 (RNA extraction method 4), and used as template in the following RT-PCRs. The cycling parameters used were as previously described (RT-PCR Method 1). The three reaction mixtures used were compiled as indicated (Table 4.2).

Table 4.2 Reaction mixtures using increased $MgSO_4$ volumes in the Access RT-PCR System

Reaction Component	1	2	3
5 X AMV/Tfl Reaction buffer	1 X	1 X	1 X
dNTP mix (10mM)	0.2mM	0.2mM	0.2mM
ONDV1aa (100pmol/µL)	2µM	2µM	2µM
ONDV4aa (100pmol/µL)	2µM	2µM	2µM
AMV RT (5U/µL)	5U	5U	5U
Tfl DNA polymerase. (5U/µL)	5U	5U	5U
RNA Sample/Template	1µL	1µL	1µL
$MgSO_4$ (25mM)	1mM	1.75mM	3mM
Nuclease Free d.d.H2O	32µL	26.5µL	24µL
Total Volume	50µL	50µL	50µL

One-Step RT-PCR method 3

Further optimization of the one tube RT-PCR method was based on using concentration of $MgSO_4$ equivalent to those of $MgCl_2$ that produced positive results in the PCR reaction using pUC 18+ F.P clone. No alterations were made to the cycling parameters used in RT-PCR method 1.

The RNA template in both these reactions, was genomic RNA isolated from sample V11. A field strain that showed positive RT-PCR results using primers specific for the fusion protein cleavage site in NDV. The reaction mixtures were as indicated (Table 4.3)

Table 4.3 **Reaction mixtures for the Access RT-PCR System using increased MgSO4 concentrations.**

Reaction Components\ Tube Number	1	2
5 X AMV/Tfl reaction buffer	1 X	1 X
dNTP mix (10mM)	0.5mM	0.5mM
ONDV1aa (100pmol/µL)	2µM	2µM
ONDV4aa (100pmol/µL)	2µM	2µM
$MgSO_4$ (25mM)	4.75mM	5mM
AMV reverse Transcriptase (5U/µL)	5U	5U
Tfl DNA polymerase (5U/µL)	5U	5U
RNA Sample	5µL	5µL
Nuclease free ddH^2O	20.5µL	20µL
Total volume	50µL	50µL

One-Step RT-PCR method 4

In an attempt to increase the efficiency of the RT-PCR, it was decided to include a primer/template-annealing step prior to reverse transcription, according to the manufacturers recommendations. The following reagents were assembled (Table 4.4).

Table 4.4 Components assembled for the template primer annealing reaction. The RNA template used in all the reactions was isolated from sample V11.

Reagent\ Tube	1	2	3	4
RNA	0.38µL	0.75µL	1.5µL	2.0µL
Nuclease free ddH_2O	1.31µL	0.94µL	0.19µL	-
ONDV1aa (100pmol/µL)	13.14µM	13.14µM	13.14µM	13.14µM
ONDV4aa (100pmol/µL)	13.48µM	13.48µM	13.48µM	13.48µM
Total Volume	15µL	15µL	15µL	15µL

The mixture was heated to 70°C for 5 minutes, followed by incubation on ice for 5 minutes. Subsequently, the following components were added to the annealed primer/template complex in the order shown (Table 4.5).

Table 4.5 Reaction components assembled for RT-PCR following primer/template annealing

Reagent/Tube	1	2	3	4
Annealed primer/template	15µL	15µL	15µL	15.31µL
5X AMV/Tfl reaction buffer	0.5 X	0.5 X	0.5 X	0.5 X
DNTP mix (10mM)	0.5µM	0.5µM	0.5µM	0.5µM
MgSO4 (25mM)	3.25mM	3.75mM	4.25mM	4.75mM
AMV RT (8U/µl)	24U	24U	24U	24U
Tfl DNA Polymerase	5U	5U	5U	5U
Nuclease free dd H_2O	17µL	16µL	15µL	14µL
Total volume	50µL	50µL	50µL	50µL

The reaction was allowed to proceed at 42°C for 60 minutes, followed by denaturation at 94°C for 2 minutes. Five cycles of amplification were performed at 94°C for 30 seconds, 55°C for 30 seconds 72°C for 1 minute and 30 seconds. A second round of amplification was performed for 35 cycles at 94°C for 30 seconds, 48°C for 30 seconds and 72°C for 2 minutes. Final extension was performed at 68°C for 7 minutes.

One-Step RT-PCR METHOD 5

The following conditions were used to amplify RNA extracted (RNA isolation method 4) from two known NDV strains V11 and LaSota. RT-PCR was performed as previously described. Reverse transcription was performed at 48°C for 60 minutes followed by denaturation at 94°C for 2 minutes. Five cycles of amplification were carried out at 94°C for 30 seconds, 40°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds. Further amplification was performed at 94°C for 30 seconds, 48°C for 30 seconds and 72°C for 2 minutes in a thermal cycler (Perkin-Elmer, USA) set for 35 cycles. Final extension was performed at 68°C for 7 minutes. The reaction mixture was compiled as indicated (Table 4.6).

Table 4.6 RT-PCR reaction components with the inclusion of RNase inhibitor.

Reagent/Tube	1(V11)	2(LaSota)	3(Control)
RNasin (30U/μL)	21U	21U	21U
5 X AMV/Tfl Reaction buffer	1 X	1 X	1 X
dNTP mix (10mM)	0.5mM	0.5mM	0.5mM
ONDV1aa (100pmol/μL)	1μM	1μM	1μM
ONDV4aa (100pmol/μL)	1μM	1μM	1μM
$MgSO_4$(25mM)	4.75mM	4.75mM	4.75mM
AMV RT (5U/μL)	5U	5U	5U
Tfl DNA Polymerase (5U/μL)	5U	5U	5U
RNA sample	5μL	5μL	2μL
Nuclease free ddH_2O	19.3μL	19.3μL	16.7μL
Total volume	50μl	50μl	50μl

RNasin, an RNase inhibitor was added to the RT-PCR reaction mixtures. This method was repeated approximately three times with no alterations in reagent composition or cycling conditions. Inconsistent results were obtained upon repetition, therefore the following alterations were made to the protocol. A positive control reaction using the clone, pUC 18 + NDV Fusion protein gene (F.P.) as template was assembled (Table 4.7).

The reaction components for the amplification of RNA extracted from known NDV field strains V10, V11, and LaSota were kept as indicated (Table 4.6).

The reaction mixture containing the clone pUC 18 + F.P. was only subjected to amplification at the end of reverse transcription signified by denaturation at 94°C for 2 minutes. Reverse transcription conditions were maintained at 48°C for 60 minutes followed by 5 cycles of amplification at 94°C for 30 seconds, 40°C for 30 seconds, 72°C for 1 minute and 30 seconds. Further amplification was performed at 94°C for 30 seconds, followed by annealing at 48°C for 30 seconds and extension at 72°C for 2 minutes, repeated for 35 cycles. Extension was finally performed at 68°C for 7 minutes.

Table 4.7 Reaction mixture alterations to RT-PCR method 5 (for the positive control clone).

Reaction mixture/tube number	1
10 X PCR buffer	2 X
dNTP mix (10mM)	5mM
ONDV1aa (100pmol/μL)	1μM
ONDV4aa (100pmol/μL)	1μM
Taq Polymerase (5U/μL)	5U
pUC 18 + F.P.	1μL
Nuclease free dd H_2O	35μL
Total volume	50μL

The clone pUC 18 + F.P. was included in order to determine whether RT-PCR failure was due to the lack of reverse transcription, or lack of PCR amplification.

Upon repetition of the same process conditions and mixtures, negative results were obtained.

One-Step RT-PCR METHOD 6

Amplification of pUC 18 + F.P. clone gave an indication that lack of amplification of the field strains may be due to the lack of reverse transcription. This led to the inclusion of an annealing step prior to reverse transcription. RT-PCR was performed using as template genomic RNA isolated from the positive control strain LaSota, from freeze-dried samples in all five reactions.

The reaction mixtures compiled for the annealing step were as indicated (Table 4.8)

Table 4.8 Reaction components assembled for the initial primer/template annealing step prior to amplification

Reaction component/tube	1	2	3	4	5
RNA/LaSota	1µL	2µL	3µL	4µL	5µL
ONDV4aa (100pmol/µL)	0.4µM	0.8µM	1.2µM	1.6µM	2µM
Nuclease free ddH_2O	13.8	12.6	11.4	10.2	9
Total volume	15µL	15µL	15µL	15µL	15µL

Template/primer annealing was allowed to proceed as previously described. The reaction components for RT-PCR were compiled as indicated (Table 4.9). A volume of 15µL of the annealed primer/template mix was added to the RT-PCR reaction mixture.

Table 4.9 Reaction components of RT-PCR reactions after performing primer/template annealing

Reagent/Tube	1
5 X AMV/Tfl DNA Polymerase	0.5 X
dNTP mix (10Mm)	0.5mM
RNasin (30U/μL)	21U
ONDV1aa (100pmol/μL)	2μM
$MgSO_4$(25mM)	4.75mM
AMV RT (5U/μL)	5U
Tfl DNA Polymerase (5U/μL)	5U
Nuclease free dd H_2O/μL	14.3
Total volume/μL	50

The RT-PCR cycling conditions were as previously mentioned (Method 5).

Two-Step RT-PCR: METHOD 7 (cDNA PRODUCTION)

Inconsistent RT-PCR results were obtained with the methods previously described (One-Step RT-PCR methods 1-6). As an alternative to one step RT-PCR, a two-step method in which reverse transcription and PCR amplification are performed separately was attempted.

A cDNA synthesis kit was used (cDNA Synthesis Kit, Boehringer Mannheim). The reaction was performed in duplicate, with one pair of tubes containing random primers $pd(N)_6$ and the other of $pd(T)_{15}$ to initiate amplification. The reaction components for the first strand synthesis were assembled in a microcentrifuge tube on ice by the addition 4μL Buffer I, 1μL of RNase inhibitor, 0.2 mM deoxyribonucleotide triphosphates, 0.04 A_{260U} $pd(N)_6$, and pd $(T)_{15}$ respectively, 2μL AMV Reverse Transcriptase, and 5μL RNA template (LaSota). After brief mixing, the mixture was collected by centrifugation and reverse transcription performed in a thermal cycler (Perkin-Elmer, USA) set at 42°C for

60 minutes, according to the manufacturer's guidelines (Boehringer-Mannheim, Germany).

Thereafter, the following reaction components were added into the microcentrifuge tube, 40μL buffer II, 1μL RNase H, 5μL *E. coli* DNA Polymerase 1 and redistilled H_2O up to a volume of 100μL. The reaction mixture was mixed and collected by centrifuging as before. Seconds strand synthesis was performed in a thermal cycler set at 12°C for 60 minutes, 22°C for 60 minutes and extension at 65°C for 10 minutes. Amplification was terminated by the addition of 10μL EDTA (0.2mM, pH 7.2), and 2μL sarkosyl solution (10%v/v)

cDNA Extraction

cDNA was purified from other synthesis reagents by phenol/chloroform extraction and subsequently concentrated by ethanol precipitation.

Briefly, cDNA was extracted according to the method described in Ausubel *et al.*, (1997). DNA extraction was performed by the addition of an equal volume of 25/24/1 (v/v/v) phenol/chloroform/isoamyl alcohol. After vigorous mixing for 10 seconds, the mixture was centrifuged at 5 X 1000g for 15 seconds at room temperature (Biofuge 13, Heraeus Instruments, Germany) to allow separation of the different components. The top (aqueous) DNA containing phase was transferred to a new tube and a 1 in 10 volume of 3M sodium acetate, pH5.2 added. DNA precipitation was accomplished by the addition of 2.5 volumes of ice-cold 100% ethanol (calculated after salt addition), and incubated on ice for approximately 5 minutes.

The DNA was pelleted by centrifugation at 14 X 1000g for 5 minutes and the supernatant discarded. This precipitation step was repeated. The pellet was washed with 1ml 70% ethanol and centrifuged as above. The DNA pellet was dried under vacuum on a rotary evaporator (Savant, USA), and resuspended in 20μL nuclease free dd H_2O.

Amplification of cDNA using PCR

The cDNA produced in the previous section using either random hexamers or oligo $(dT)_{15}$ was used as template for the amplification of the 75% region of the fusion protein. Each of the cDNA solution was divided into two separate reaction mixtures. The first reaction was compiled to include, 2 X PCR buffer containing 15mM $MgCl_2$, 0.2mM dNTPs, 2μM each primer (ONDV1aa and ONDV4aa), 1μL of cDNA, 5U Taq Polymerase, and 35μL nuclease free ddH_2O. The second mixture was the same in all respects to the first, except that the 2 X PCR buffer used lacked $MgCl_2$. $MgCl_2$ was from a (25mM) to reach a final concentration of 4.75mM and nuclease free ddH_2O added to bring the total reaction volume to 50μL.

The four reaction mixtures were subjected to amplification with a "hot" start in a thermal cycler set at 94°C for 5 minutes, followed by 5 cycles of amplification at 94°C for 5 minutes, 55°C for 30 seconds, and extension at 72°C for 1 minute. This was followed by 30 cycles of amplification at 94°C for 45 seconds, 48°C for 1 minute, 72°C for 3 minutes and finally extension at 72°C for 7 minutes.

Purification of PCR products

Of the four reactions tested the amplimer of interest was obtained from the cDNA produced using random hexamers $p(DN)_6$, and amplified using 4.75mM $MgCl_2$. Although the band of interest was present, it was contaminated with primer dimers and non-specific amplimers of varying molecular weight. It thus became necessary to isolate the required band.

The NucleoSpin Extract 2 in 1 Kit was used for the purification process. Briefly, 400μL of buffer NT2 was mixed with the sample, the mixture added onto a NucleoSpin Tube and forced though the column (6 X 1000g for 60 seconds), (Biofuge 13, Heraeus Instruments, Germany).

The eluent was discarded, and 700μL of the ethanol containing buffer (NT3) added to the column and centrifuged (14 x 1000g for 60 second). The flow-through was discarded

and this washing step repeated. Once the flow-through from the final washing step was removed, quantitative amounts of buffer NT3 were removed by centrifugation (14 X 1000g for 60 seconds). Finally the DNA was eluted from the NucleoSpin tube by the addition of 50µL of elution buffer and centrifugation (14 X 1000g for 60 seconds), and collected in a clean 1.5ml centrifuge tube, according to the manufacturer's instructions.

Two-Step RT-PCR: METHOD 8 (Lomniczi, personal communication)

An alternative RT-PCR protocol to the cDNA kit mentioned previously entailed the use of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), in a separate reverse transcription reaction, followed by PCR amplification using Taq Polymerase.

Two separate reaction volumes were prepared (Table 4.11)

Table 4.10 **Reaction mixtures compiled for the reverse transcription of RNA isolated from LaSota using M-MLV RT.**

Reaction Mixture/Tube number	A	B
5 X M-MLV Reaction buffer	1 X	1 X
Random hexamers $p(dN)_6$ (10pmol/µL)	1µM	1µM
M-MLV RT (200U/µL)	200U	200U
RNA Template (LaSota)	5µL	5µL
dNTP's (10 mM)	0.2mM	0.5mM
RNasin (25U/µL)	25U	12.5U
Nuclease free ddH_2O	8µL	8µL
Total Volume	25µL	25µL

Reverse transcription was performed at 37°C for 90 minutes, followed by enzyme inactivation at 98°C for 5 minutes, and finally incubation on ice for 15 minutes (Lomniczi., personal communication)

A 5µL volume of each of the above prepared cDNA mixtures were used as templates in PCR amplification reactions. Both reaction mixtures contained 1X PCR buffer without $MgCl_2$, 1mM $MgCl_2$, 0.1µM dNTP's, 0.1µM BSA, 0.3µM of each of the primers

ONDV1aa, and ONDV 4aa, 2U Taq Polymerase, and nuclease free ddH_2O to a volume of 100µL (Lomniczi personal communication).

Amplification was performed in a thermal cycler set at 5 cycles of 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 1 minute, and 30 cycles at 94°C for 45 seconds, 48°C for 1 minute, and 72°C for 3 minutes. Final extension was performed at 72°C for 10 minutes (Lomniczi personal communication).

Optimisation of the Two-Step RT-PCR METHOD 8

RNA was extracted from the positive control strain LaSota according RNA extraction method number four and used as template in the optimization schedule (Tables 4.11 (A), and 4.11 (B)).

Reverse transcription was performed as described above without any alterations. The reaction was performed in five identical reactions in order to produce enough template for the optimization schedule.

PCR optimization was based on modified Taguchi methods (Cobb & Clarkson, 1994).

Table 4.11 (A) Taguchi based PCR optimisation schedule using PCR buffers without $MgCl_2$. The values listed indicate the final concentration of each reagent in a total reaction volume of 100µL

Reagent 1/tube number	1	2	3	4	5	6	Reagents 2
10 X PCR buffer without $MgCL_2$	1 X	1 X	1 X	1 X	1 X	1 X	
BSA (10mg/ml)	0.1µg/µg	0.1µg/µg	0.1µg/µg	0.1µg/µg	0.1µg/µg	0.1µg/µg	
$MgCl_2$ (25mM)	2.5mM	2.5mM	2.5mM	2.5mM	2.5mM	2.5mM	
dNTPs'(10pmol/µL)	0.1µM	0.5µM	0.8µM	0.8mM	0.5mM	0.1mM	(10mM)
ONDv1aa (100pmol/µL)	0.2µM	0.3µM	0.6µM	6µM	3µM	2µM	(100pmol/µL)
ONDV4aa (100pmol/µL)	0.2µM	0.3µM	0.6µM	6µM	3µM	2µM	(100pmol/µL)
Taq Polymerase (5U/µL)	2U	2U	2U	2U	2U	2U	
cDNA mix (µL)	5	8	10	10	8	5	
Nuclease free ddH_2O	68.6	59.6	48.6	48.6	59.6	68.6	
Total volume/µL	100	100	100	100	100	100	

Reactions 4, 5, 6 were compiled by the addition of the reagents listed in reagent column 2, instead of those listed in reagent column 1.

Table 4.11 (B) Taguchi optimisation schedule using PCR buffer containing $MgCl_2$. The values indicate the final concentrations of each reagent in a reaction volume of 100µL.

Reagent 1/ tube number	1	2	3	4	5	6	Reagent 2
10 X PCR buffer with MgCl2	1 X	1 X	1 X	1 X	1 X	1 X	
BSA (10mg/ml)	0.1µg/µL	0.1µg/µL	0.1µg/µL	0.1µg/µL	0.1µg/µL	0.1µg/µL	
dNTPs' (10pmol/µL)	0.1µM	0.5µM	0.8µM	0.8mM	0.5mM	0.1mM	(10mM)
ONDV1aa (10pmol/µL)	0.2µM	0.3µM	0.6µM	6µM	3µM	2µM	(100pmol/µL)
ONDV4aa (100pmol/µL)	0.2µM	0.3µM	0.6µM	6µM	3µM	2µM	(100pmol/µL)
Taq Polymerase (5U/µL)	2U	2U	2U	2U	2U	2U	
cDNA mix	5	8	10	10	8	5	
Nuclease free ddH_2O	78.6	69.6	58.6	58.6	69.6	78.6	
Total volume/µL	100	100	100	100	100	100	

Reactions 4, 5, 6 included the addition of the reagents listed in reagents column 2, instead of those listed in reagent column 1.

PCR cycling conditions were maintained at 5 cycles of 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 1 minute, and 30 cycles of 94°C for 45 seconds, 48°C for 1 minute, 72°C for 2 minutes and finally extension at 72°C for 10 minutes.

A 5μL volume of each sample was mixed with 2μL loading buffer (200μg bromophenol blue, 32g sucrose), loaded on a 1% (w/v) agarose gel stained in ethidium bromide (0.5μg/mL) and separated by electrophoresis in TAE buffer (40mM Tris.acetate, 2mM EDTA, pH 8.3).
The DNA was separated by electrophoresis and visualized by UV-transillumination (Biotron Germany, Spectroline Transilluminator, USA.)

4.4.2 MATRIX PROTEIN GENE AMPLIFICATION (nt 44-1141)

cDNA Production/ Reverse Transcription (RT)

cDNA template (optimised two-step RT-PCR method number eight) was used as template to amplify a region comprising 88% of the matrix (M) gene, of the field strains under investigation. A 1097bp product was amplified using the primers:

M1 (R) 5'-TCT AGG ACA ATT GGG CTG TAC TTT GAT T –3'(M gene nt 44-71)

M2 (F) 5'-AGA GAC GCA GCT TAT TTC TTA AAA GGA TTG –3'(M gene nt 1112-1141)

(Herczeg *et al.*, 1999)

Amplification was performed in a 100μL reaction containing, 1X PCR buffer (without $MgCl_2$), 1.5mM $MgCl_2$, 1 pmol/μL dNTPs, 0.1μg/μL BSA, 1 pmol/μL of each of the primers (M1 and M2), 2U Taq Polymerase and 5μL cDNA mix. The amplification conditions were set for 35 cycles at 94°C for 45 seconds, annealing at 57°C for 1 minute, 72°C for 2 minutes. Final extension was performed at 72°C for 10 minutes. (Lomniczi, personal communication).

A 5μL volume of each of the amplification products were analysed by electrophoresis (Biotron Germany, Spectroline Transilluminator, USA.) on a 1 % (w/v) ethidium bromide stained agarose gel

Purification of PCR Products

The NucleoSpin Extract Kit was used to purify PCR products of the desired size. The method was performed without any alterations to that previously described. The final product was eluted with 30μL elution buffer NE. Aliquots of 5μL of each of the purified samples were again analysed by agarose gel electrophoresis. In order to ensure that all the undesired products had been removed.

4.4.3 FUSION PROTEIN CLEAVAGE SITE AMPLIFICATION

PCR 1: (nt M Gene 778-F Gene nt 545)

The sequence from matrix protein gene nucleotide 1163 to fusion protein gene nucleotide 470 was amplified in two separate PCR reactions.

A master mixture was assembled and aliquoted into each PCR tube to be used so that each 100μL volume contained 1X PCR buffer (10 X), 2.5 mM $MgCl_2$,, 0.1μM BSA, 2U Taq Polymerase, 5μL cDNA mix, and 2μM of each of the primers:

K1 (R) 5'-GGG RAA GAR AGT GAC WTT TGA CA-3'(M gene nt 778-800)
K2 (F) 5'-TKG GAT AAW CCR YYR GTG ACC TC-3'(F gene nt 523-545)

(Herczeg *et al.*, 1999)

Amplification was performed for 5 cycles at 94°C for 45 seconds, 55°C for 30 seconds, and at 72°C for 1 minute. Further amplification was performed for 35 cycles set at 94°C for 45 seconds, 48°C for 1 minute and extension at 72°C for 3 minutes. Further extension was performed at 72°C for 10 minutes (Lomniczi, personal communication).

PCR Coring

The primary product, 1008bp in size, flanked by primers K1 and K2 was used in a nested PCR to amplify a 557bp amplimer. The products of the first PCR reaction were contaminated with non-specific amplimers, which necessitated the isolation and purification of the product of interest. This was achieved by a PCR coring technique (Rybicki, 1992). Briefly, alternate lanes on a 1% ethidium bromide stained agarose gel were loaded with ~10μL sample, and separation allowed under 90-100V for approximately 1hour. Excess gel fragments flanking the band of interest were excised using a sterile blade, under a long-wave hand-held UV light and discarded. The sample lane flanked by marker DNA was cut out and marked to ensure re-orientation with the remainder of the gel. The excised piece was viewed under long wave UV light and the band of interest in the sample lane was indicated by "stabbing". Using gloves and a sterile blade approximately 5mm of the tip of a sterile Gilson p10 (white) pipette tip were removed, to produce core samplers. The marked segment was re-aligned with the remainder of the gel. "Core samplers" were used to stab out the cores of agarose from the center of the bands of interest using the stabbed gel as reference. The gel was viewed once coring was complete to ensure that correct samples were collected. A volume of 10μL was collected for each core. These cores were subsequently used in this form as substrate in PCR reactions (Rybicki, 1992).

Nested PCR: (nt M Gene 1163- F Gene nt 470)

Nested PCR for the amplification of a 557bp product was performed using the primers:

MV1 (R) 5'-CCY RAA TCA YYR YGR YRC YRG ATA A-3' (M gene nt 1163-1187)

B2: (F) 5'-KCR GCR TTY TGK KTG GCT KGT AT-3' (F gene nt 470-492)

(Herczeg *et al.*, 1999)

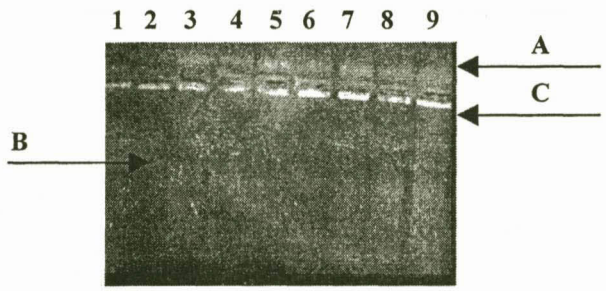
A 100μL PCR reaction mixture containing 2.5 mM $MgCl_2$, 0.1mM dNTPs, 0.1μM BSA, 2μM of each of the primers MV1 and B2, 2U Taq Polymerse, 1 X PCR Buffer (10 X) was assembled.

Amplification was performed in a thermal cycler set at 20 cycles of 94°C for 45 seconds, 52°C for one minute, and 72°C for 2 minutes. Final extension was performed at 72°C for 10 minutes (Lomniczi, 2000, personal communication)

Subsequent to amplification, reaction products were analysed on a 1% (w/v) agarose gel stained in ethidium bromide. Amplimers of interest were then extracted using the NucleoSpin Extract Kit.

4.5 RESULTS AND DISCUSSION

Genomic RNA (RNA extraction method 1) extracted from the positive control strain LaSota was analysed by electrophoresis (Biotron Germany, Spectroline Transilluminator, U.S.A.) on a 1% (w/v) agarose gel containing ethidium bromide.



Figurer 4.1 RNA extracted from allantoic fluid using RNA extraction method 1. The bands were separated on a 1% (w/v) agarose gel stained with ethidium bromide. Lanes 6-9 were loaded with 5µL RNA sample, lanes 1-5 contained 10µL extracted RNA.

Bands indicated by arrow A, migrate towards the negative pole (Figure 4.1). These molecules are thought to be proteins, or other positively charged debris released during the extraction process. Arrow C, located below the wells indicates a band indicative of the desired RNA. The genomic RNA has a molecular weight of approximately 15kb, these molecules are very large and do not migrate far into the gel (Figure 4.1).

The brightness of the bands varied between those loaded with 5µL (lanes 1-4) and those that contained 10µL of extracted RNA (Lanes 5-9) (Figure 4.1). Those containing 10µL

(Lanes 5-9) had greater fluorescence than those loaded with 5μL (Lanes 1-4) (Figure 4.1). Arrow B, highlights an unknown contaminant present in the majority of the samples. These molecules migrate towards the positive pole, and could include reagent molecules used during the extraction process. These molecules do not display a high degree of fluorescence. They are not nucleic acid, but could be LiCl salts or contaminating debris from the isolation process (Wallace, 1987).

For use in RT-PCR, intact full length RNA had to be extracted from virion particles. RNA was extracted from all the field strains obtained for use as template in RT-PCR. However, the state or condition of the extracted RNA was not known. Spectrophotometric determination of the concentration of RNA extracted from allantoic fluids would only serve to confirm the presence or absence of RNA. RNA extracted using the rest of the methods described in this section, was therefore not analysed spectrophotometrically. As the deduced concentration values obtained would not inform us whether we had successfully isolated intact full length genomic RNA this could only be determined from RT-PCR results.

4.5.1 ONE STEP AMPLIFICATION OF THE FUSION PROTEIN GENE (NT 334-1682)

Method 1

In this section a 75% region of the NDV fusion protein gene flanked by primers ONDV4aa (F) and ONDV1aa (R) was amplified using RT-PCR. RNA extracted from the LaSota control strain was amplified using the Access RT-PCR Kit. The Access RT-PCR Kit (Promega, USA) combines two previously separate procedures, reverse transcription, and the Polymerase Chain Reaction (PCR) in a single tube reaction, optimized for both enzymes.

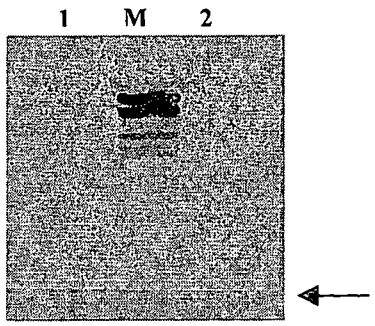


Figure 4.2 RT-PCR amplification results obtained after the amplification of a 75% region of the NDV fusion protein gene using the Access RT-PCR Kit. A 1% ethidium bromide stained agarose gel, shown in lanes 1&2 experimental results obtained; M: Lambda DNA Marker (EcoR I and Hind III digested). The arrow indicates the presence of primer dimers present in sample reaction 1 & 2.

Negative results were obtained using the One Step RT-PCR method 1 (Figure 4.2). Sequence specific primers were used for reverse transcription and PCR. The only nucleic acid detected in the reactions was that indicative of primer dimers. Primer dimers are double stranded PCR artifacts, that consist of the two primers and their complementary sequences (Watson, 1989). These non-specific products are formed when one primer is extended using the other as template. Primer dimer formation is dependent on the concentration and sequence of the primers, and is commonly seen in PCRs with a high number of cycles. (Arnheim & Erlich, 1992). This band migrated ahead of the dye front and its size was much smaller than that of the expected fragment.

The absence of the amplification product of interest (Figure 4.2) could be attributed to a number of factors known to affect the effectiveness of RT-PCR. These include, the presence of RNA degrading enzymes, the presence of inhibitory agents such as SDS, NaCl, or guanidine thiocyanate. These chemicals may have been carried over from the RNA extraction methods used (Promega Technical Bulletin, USA).

RT-PCR is a highly sensitive technique. It has however, been observed that when transferring protocols between different thermocyclers, or during the development of

novel applications, a range of reaction mixtures have to be optimized in order to obtain maximum yield, increase specificity and reaction fidelity (Cobb, & Clarkson, 1994).

It was therefore decided to optimize the magnesium ion concentration in a reaction series containing magnesium chloride in increasing levels. Increments were made by the addition of 0.75mM, 1.75mM, 3.75mM, and 4.75mM of a 25mM $MgCl_2$ stock into a 50μL PCR reactions using, Taq polymerase to amplify the sequence of interest using. A plasmid (pUC 18) containing the entire NDV fusion protein gene (F. P) was used as template.

Method 1: Optimisation

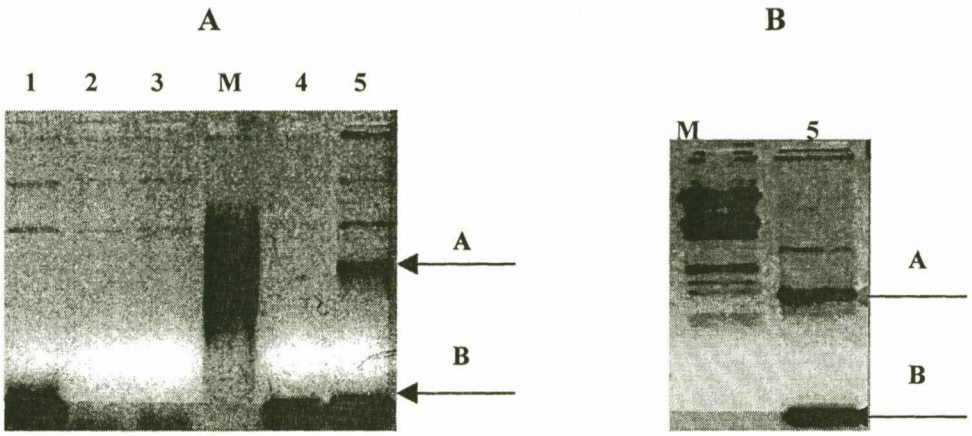


Figure 4.3 Schedule used for magnesium ion concentration optimization for PCR amplification using pUC 18 + F.P. as template. PCR reactions were performed using various concentration of Mg^{2+} to determine the optimum level required for amplification of a 1349bp amplicon. A 5% aliquot of the reaction products, was used for analysis by agarose gel electrophoresis on an ethidium bromide stained 1% (w/v) agarose gel. **Panel A**: M: Lambda DNA ladder, lane 1: 10 X PCR buffer containing 15mM Mg^{2+}, lane 2: 0.75mM Mg^{2+}, lane 3: 1.75mM Mg^{2+}, lane 4: 3.75mM Mg^{2+}, lane 5: 4.75mM Mg^{2+}. **Panel B**: lane M: Lambda DNA ladder, lane 5: 4.75mM Mg^{2+}. **Key**: arrows **A,** amplification products of interest ~1349bp; arrows **B,** primer dimers.

The plasmid vector pUC 18 (2.69kb) containing the entire fusion protein gene of LaSota was visible in all five amplification reactions (Figure 4.3). Two forms of the plasmid were observed, the more compact supercoiled form that migrates further in the gel. The closed circular form migrates over a shorter distance in the gel as it is retarded because of its shape (Brock & Madigan, 1991). Primer dimers were observed, in high concentration,

in all five reactions, migrating ahead of the dye front as indicated by the bottom arrows (Figure 4.3, arrow B). The amplification product of interest was only evident in reaction five. This band was of the expected size ~1349bp in size (Fig 4.3, arrow A).

No amplification products were observed at low Mg^{2+}concentration, inclusive of reaction number one where Mg^{2+} containing buffer was used. High yields of the amplification product were obtained at high concentrations of Mg^{2+} (4.75mM) in reaction five (Figure 4.3, arrow A)

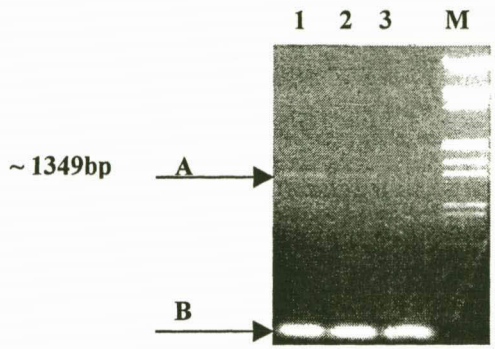


Figure 4.4 RT-PCR Amplification of a 1349bp region of the fusion protein gene of NDV strain V11. RT-PCR cycling was performed at varying concentrations of Mg^{2+}. Lane 1: 1mM Mg^{2+}, lane 2: 1.75mM Mg^{2+}, lane 3: 3mM Mg^{2+}, M: lambda DNA marker.

Method 2

The above figure displays the results obtained using the One-Step RT-PCR method 2. The cycling parameters used were the same as those used in One-Step RT-PCR method 1. The RNA template used in the above reactions was isolated from a freeze-dried sample of a known NDV strain V11 (own designation). Low yields of the amplification product were observed in all three reactions (Figure 4.4, arrow A). Amplification was also observed at a magnesium ion concentration of 1mM, which is the same level as that used in One-Step RT-PCR method 1. The yield of product in all three reactions, at varying levels of Mg^{2+} was the same. The intensity of the bands produced in all the reactions remained constant, as indicated by the intensity of fluorescence of these bands (Figure 4.4, Arrow A).

High concentrations of primer dimers were present in all cases (Arrow B), as indicated by the high degree of fluorescence displayed by these bands (Figure 4.4).

It has been reported that in order to obtain a positive RT-PCR signal, using sequence specific primers to amplify a 254bp fragment within the fusion protein gene, at least 10^5 $ELD_{50/ml}$ of NDV particles should be present in the allantoic fluid (Kant *et al.*, 1997). In this particular case a larger fragment of the fusion protein gene (1349bp) is to be amplified using a different primer pair (ODV1aa and ONDV4aa), the template used in both cases is genomic NDV RNA. The low product yields obtained could have resulted due to the use of low concentrations of starting material. The concentration of NDV particles in the freeze-dried samples was unknown.

Method 3

RT-PCR amplification using the Access RT-PCR Kit was repeated using NDV (V11) genomic RNA as template. The cycling conditions used were those mentioned in the One Step RT-PCR method 1. Two reaction mixtures were compiled by the addition of different magnesium chloride levels, one containing 4.75mM $Mg^{2+}$and the other 5mM Mg^{2+}. No further alterations were made to the reaction components compiled in the RT-PCR method 2. No amplification product was obtained in both reactions. Even though the magnesium ion concentration used was the same as that which resulted in positive amplification of the amplicon of interest, using the pUC 18 + F. P. clone as template.

A final Mg^{2+} concentration of 4.75mM was obtained from determining optimal Mg^{2+} levels required for the amplification of this sequence from the cloned fusion protein gene (pUC 18 + F.P.) using Taq polymerase (Boehringer Mannheim). In the One Step RT-PCR method 2 procedure, the RNA template used was extracted from a freeze-dried sample of a known NDV strain (V11). When the same level of magnesium ions was used in the RT-PCR, no amplification products were obtained. The failure of the RT-PCR could be attributed to the fact that the freeze-dried sample might have contained relatively lower levels of template compared to that of the clone used for optimization. Thus, the concentration of 4.75mM $MgCl_2$ obtained from the magnesium ion

optimisation schedule using the cloned fragment appears not to be applicable to the amplification of RNA extracted from known NDV field strains using RT-PCR.

In addition Taq polymerase (Boehringer Mannheim) was used for the optimisation of Mg^{2+} levels, whereas the Access RT-PCR Kit employs Tf 1 DNA polymerase for amplification. Suggesting that reaction conditions e.g. Mg^{2+} levels are not interchangeable between different polymerases/enzyme systems used, and that optimization may have to be performed for each template/enzyme system. The formation of primer dimers could also have led to a decrease in the specificity for the sequence of interest.

Method 4

RT-PCR method number 4 involved the inclusion of a primer/template annealing step prior to RT-PCR amplification. This annealing step should reduce the formation of primer dimers, thereby restoring the sensitivity of the process (Arnheim & Erlich, 1992). Annealing of primers to the template prior to PCR amplification also increases the efficiency of amplification by destroying inhibitory secondary RNA structures (Sambrook *et al.*, 1989). The RNA template (V11) was denatured by heating to 70°C in the presence of both primers for five minutes. Primer annealing was allowed to proceed by incubating the mixture on ice for five minutes, according to the manufacturer's instructions (Promega, USA).

Reverse transcription was performed at a lower temperature as that used initially, 42°C for a period of 60 minutes. In the previous reactions reverse transcription was performed at 48°C for 45 minutes. Thus reverse transcription was performed at less stringent conditions for a longer period in order to increase the probability of primers binding to the template. An additional extension step at 68°C for 7 minutes was also included. No amplification products were obtained using this protocol (results not shown).

Method 5

The following alterations were made to the protocol used previously, RNasin® a ribonuclease inhibitor (Promega, USA) which inhibits the degradation of the RNA template by endogenous RNA degrading enzymes (RNases) was included. RNasin® was added to all the reactions below (Figure 4.5) at a concentration of 21U. The annealing temperature of the first five cycles was decreased to 40°C, as compared to that used previously of annealing at 55°C. Annealing at 40°C is less stringent, and should therefore allow more frequent priming. The desired amplification product of ~1349bp, was present in both experimental reactions (Figure 4.5).

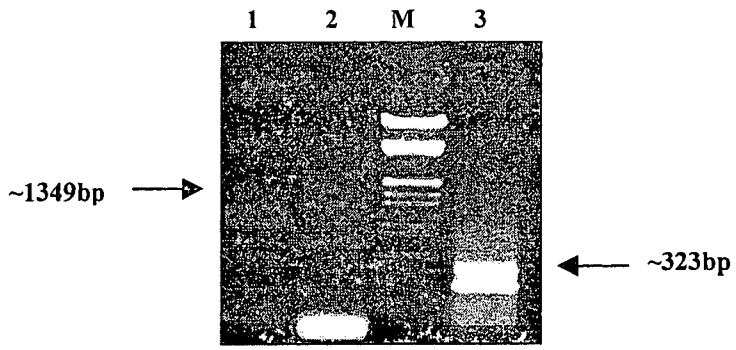


Figure 4.5 RT-PCR Amplification in the presence of an RNase inhibitor. Lane 1: (freeze-dried) strain V11, lane 2: LaSota , M Lambda DNA marker, lane 3: Access RT-PCR Kit positive control reaction.

In lane three (Figure 4.5) the arrow indicates a 323bp fragment of the positive control reaction. A smaller non-specific amplification product is also visible below that of the desired size. Annealing at a low temperature, 40°C for 30 seconds, is less stringent than those used previously, 55°C for 1 minute for the first five cycles of amplification. This allows the primers to bind in a non-specific manner to the RNA template, hence the presence of smaller contaminating bands in both the control (Lane 3) and experimental reactions (Lanes 1 & 2) (Figure 4.5). The product of interest is present at very low yields when amplifying RNA from LaSota and V11, indicating that unlike it was previously thought, the concentration of the initial RNA template, may not be the major factor affecting amplification, as the LaSota vaccine strain contained a higher concentration of RNA containing virions, than that of the freeze-dried sample.

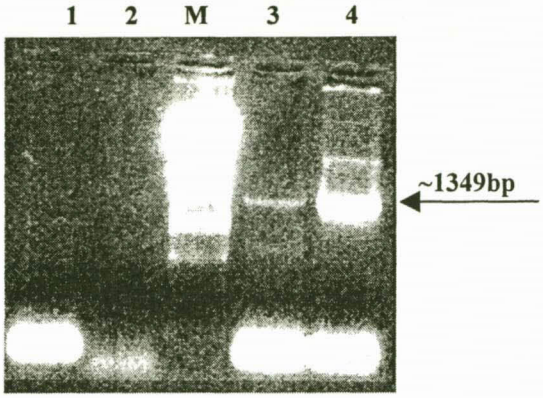


Figure 4.6 Altered RT-PCR method number 5. Lane 1: V31 genomic RNA, lane 2: V10 genomic RNA, M: Lambda DNA ladder, lane 3: LaSota genomic RNA, lane 4: pUC 18 + F.P. gene.

RT-PCR method number 5 was repeated to assess its reproducibility, with the inclusion the positive control clone pUC 18 + F.P. The cycling parameters used were unaltered. Positive results were observed for the positive control strain LaSota, and the pUC 18 + F.P. clone. The intensity of the band representing the band of interest in the LaSota reaction mix was, low when compared to that observed with the pUC 18 + F.P. clone.

Reactions using the RNA template of freeze-dried samples of known NDV strains V10 and V30 gave no amplification products, even in the presence of 21U RNase inhibitor. This RT-PCR was repeated a few times, however, no amplification products were obtained with RNA extracted from freeze-dried samples of strains V10, and V31. It appears that amplification is inhibited in the presence of low levels of RNA template, as is the case with strains V31 and V10. Primer dimers were evident in all the reactions performed inclusive of both the LaSota and pUC 18 + F. P. reactions.

The clone pUC 18 + F. P. was included in order to determine whether the lack of amplification was due to sub-optimal cycling parameters, or caused by some other factors. The intensity of fluorescence of the fragment produced upon amplification of the sequence of interest using the cloned fragment as template was very high. There were no other non-contaminating bands observed in this reaction (Figure 4.6, lane 4). This indicated that the thermal cycling conditions used were sufficient for PCR.

Method 6

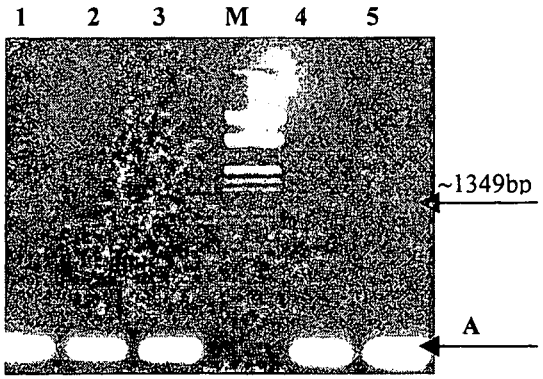


Figure 4.7 RT-PCR using LaSota genomic RNA. RNA was extracted from freeze-dried preparations of the NDV strain LaSota. Amplification was performed after annealing various levels of the forward primer ONDV4aa to the template. Amplification was performed in the presence of 21U RNasin. Lane1: 0.4μM primer, lane 2: 0.8μM primer, lane 3: 1.2μM primer, M: Lambda DNA ladder, lane 4: 1.6μM primer, lane 5; 2.0μM primer.

The following alteration were made to the protocol used in method 5, primer/template annealing was performed at varying concentrations of the forward primer ONDV4aa (2.4μM, 0.8μM, 1.2μM, 1.6μM, 2.0μM). The concentration of the reverse primer ONDV1aa was maintained at 2.0μM in all the reactions. Despite the inclusion of a template/primer annealing step prior to amplification, large amounts of primer dimers were present in all experimental reactions (Arrow A, Figure 4.5). Positive results were observed in reaction one containing 0.4μM ONDV4aa and 2.0 μM ONDV1aa, and reaction five containing ONDV4aa and ONDV1aa in equimolar amounts. Smaller non-specific amplification products were observed in reaction tube number one.

The results obtained using the One-Step RT-PCR amplification protocols attempted thus far were inconsistent. In certain cases during the optimisation process the amplicon of interest was obtained with the positive control strain LaSota (Figure 4.4, 4.6, 4.5). The product was, however, obtained in low yields as compared to that obtained for the pUC 18 + F. P. clone. Amplification using this clone was performed using Taq polymerase only, and not the reaction components assembled for RT-PCR (Figure 4.6, 4.3).

The lack of amplification products, or the production of low yields thereof can be attributed to a number of factors. Included amongst these is the use of an insufficient number of cycles. This factor is not applicable in the majority of the RT-PCR reactions performed as the amplification was performed at cycling parameters of 35 cycles. Another factor that could have contributed to the failure of RT-PCR amplification is the use of sub-optimal reaction composition, including sub-optimal magnesium ion levels. It has, however, been observed that performing RT-PCR at varying magnesium ion concentration had no visible effect on the product yield (Figure 4.4, method 3).

Amplification was also attempted at various annealing temperatures (55°C in methods 1, 2, 3, and at 40°C for methods 4, 5, 6), in an attempt to optimise the RT-PCR cycling conditions with very little or no increase in specificity for the target sequence. Non-specific amplification products resulted due to the low stringency conditions created by decreasing the annealing temperature to 40°C.

The inclusion of a final extension step at various temperatures (at 72°C in method 1, and at 68°C in methods 4, 5, 6) was also included, with no visible increase in product yield.

Interestingly, researchers attempting to apply RT-PCR to the detection of Ross River virus (RRV) in mosquitoes reported similar effects as those observed in this study. These researchers found that RT-PCR performed in a one tube system was only successful if relatively large amounts of viral RNA were present (Sellner *et al.*, 1992). In our findings this effect was observed when amplification products were obtained upon performance of RT-PCR using RNA extracted from LaSota vaccine strain (Figure 4.6 method 5, Figure 4.5 method 6), whilst no products were obtained with known NDV strains V10, V31). Sellener and co-workers (1992), also reported that the yield of the amplification product was not affected by the magnesium ion levels. In this study this was observed using method 2 (figure 4.4).

This phenomenon, whereby a loss in sensitivity is observed upon detection of viral RNA with RT-PCR is attributed to a direct molecular interaction on Taq polymerase by reverse transcriptase enzymes (Sellner *et al.*, 1992). This interference occurs when the ratio of reverse transcriptase (RT) to Taq polymerase is greater than ~3:2 (Sellner *et al.*, 1992). In the majority of the RT-PCR reactions performed thus far the RT: Taq polymerase was 1:1 and 4:1 (method 4). These researchers reported the same effect using a different RT (Moloney Murine Leukemia RT), and Taq polymerase obtained from a different supplier (Sellner *et al.*, 1992). In the Access RT-PCR system a different polymerase enzyme is used (Tfl DNA polymerase). Sellner and co-workers, (1992) did not include this enzyme in their study. The results obtained in this study indicate that this phenomenon may be true for inhibition of Tfl DNA polymerase activity by active AMV RT.

This problem can, however, be circumvented by the performance of cDNA synthesis separately before PCR amplification. Coincidentally, such a kit was readily available in the laboratory and was used.

4.5.2 TWO STEP RT PCR AMPLIFICATION OF THE FUSION PROTEIN GENE (NT 334-1682)

Method 7

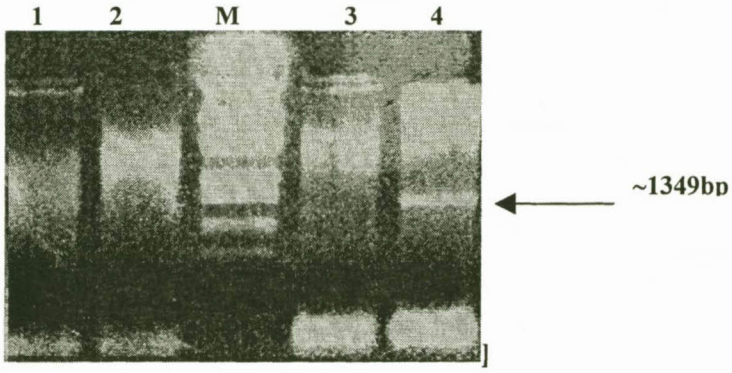


Figure 4.8 Two-step RT-PCR method number 8. cDNA was produced using AMV RT for first strand synthesis, and *E. coli* DNA polymerase 1 for seconds strand synthesis. PCR amplification was performed using 5U Taq polymerase. Lane 1: oligo dT used for cDNA synthesis and PCR amplification in 15mM Mg^{2+} containing buffer, lane 2: oligo dT used for cDNA synthesis and PCR amplification was performed using 4.75mM Mg^{2+}, M: Lambda DNA ladder, lane 3: random hexamers used for cDNA synthesis and PCR amplification performed in 15mM Mg^{2+} buffer, lane 4: random hexamers used for cDNA synthesis and PCR amplification was performed in 4.75mM Mg^{2+}.

The results displayed in the figure above are those obtained from the amplification of the fusion protein gene sequence defined by primers ONDV1aa and ONDV4aa, using a cDNA synthesis kit prior to PCR. RNA extracted from the LaSota vaccine strain was used as template in all the reactions (Figure 4.8). The cDNA produced was double stranded. This was facilitated by performing 1st strand synthesis by reverse transcription using AMV RT. The entire cDNA reaction volume was subsequently added to the reaction mixture assembled for second strand synthesis to a total reaction volume of 100μL. Second strand synthesis was performed using *E. coli* DNA polymerase I.

Performing RT-PCR as separate reverse transcription and PCR steps serves to eliminate the inhibitory effects of active reverse transcriptase enzymes on Taq polymerases (Sellner

et al., 1992). The inhibitory effect of the reverse transcriptase on Taq polymerase was avoided using this kit. It is thought that, in this method, the increment of the reaction volume, during second strand synthesis to 100μL, minimised the inhibitory effect of the reverse transcriptase (AMV RT). The dilution of the reverse transcription reaction mixture during cDNA second strand synthesis (~1:5) is assumed to have decreased the ratio of the two enzymes to levels that inhibited this phenomenon.

The cDNA produced was purified by phenol/chloroform and ethanol precipitation, thus eliminating inhibitory substances that may affect amplification by PCR.

The linear NDV RNA genome contains polyadenylation signals at the end of every gene. The use of $pd(T)_{15}$ as primers for reverse transcription seemed ineffective. Multiple non-specific bands were observed in the reactions in which these primers were used (Figure 4.8 lanes 1, 2).

PCR amplification in the presence of 4,75mM Mg^{2+}using Taq polymerase produced a good yield of the amplification product of interest (Figure 4.8). This reaction employed the use of random hexamers for reverse transcription, and good amplification results were obtained. The random hexamers will therefore be used in future for reverse transcription, instead of sequence specific primers. This concentration of magnesium ions was previously observed to produce high yields of the target sequence upon amplification using pUC 18 + F. P. as template (One-Step RT-PCR method 1 optimisation). Indicating that relatively high magnesium ion concentrations are required for the amplification of this region using Taq polymerase.

Non-specific amplification products greater than the expected band size were visible in the four reactions. The procedure followed in this technique necessitates the purification of cDNA products from other reagent components prior to PCR amplification. In addition the time required for cDNA second strand synthesis (~2hours 10 minutes), was found to increase the process time unnecessarily. It was therefore decided to pursue a less time consuming alternative.

Method 8

An RT-PCR method that involved the use of Moloney Murine Leukemia Reverse Transcriptase (M-MLV) for cDNA production, and the use of Taq polymerase (Lomniczi *et al.*, 2000, personal communication) was attempted. M-MLV reverse transcriptase functions optimally at 37°C, thus the temperature for reverse transcription was adjusted accordingly. Annealing at 55°C was used for the first 5 cycles of amplification followed by 30 cycles of annealing at 48°C.

No amplification products were obtained using this protocol. The ratio of M-MLV RT to Taq polymerase (~1:1 in a final volume of 100μL) in this case, is thought not to have led to the failure of the experiment as the reverse transcriptase was heat inactivated at 98°C for 5 minutes prior to the addition of the cDNA mix to the PCR mixture.

Previous optimisation experiments using the pUC 18 + F. P. clone and Taq polymerase highlighted the effect of sub-optimal conditions on amplification (Figure 4.3, One-Step RT-PCR method 1 optimisation). The results obtained from the above mentioned experiment indicated that relatively high magnesium ion levels were required for successful amplification. In this current method the final concentration of magnesium ions was 1mM, which is lower than those that produced the amplicon of interest in the optimisation of method 1. We therefore found it necessary to determine the optimum reaction conditions that would produce the amplicon of interest at sufficiently high yields.

Method 8: Optimisation

Two groups of reaction based on Taguchi methods were used for the optimisation process. The PCR section of the technique was the only one that was subjected to optimisation. The one group of reactions was compiled by the addition of magnesium chloride from a 25mM stock (Figure 4.9, A), whilst the other group of reactions involved the use of magnesium chloride containing buffer. No alterations were made to the RT-PCR cycling conditions mentioned in method 8.

Modified Taguchi methods were applied in this optimisation method as they allow the establishment of optimal conditions for a particular process, by using the minimal number of experiments possible (Cobb & Clarkson, 1994). Five of the reactions used produced the amplification product of interest (Figure 4.9, Lanes 6, 7, 10, 11, 12). In comparison the amplification product produced in lane 6 produced a higher product yield than those in experiments 7, 10, 11, 12 as judged from the intensity of fluorescence of the bands upon UV transillumination (Figure 4.9).

It was decided to use the reaction mixture compiled in reaction 6 for the screening of the field isolates under investigation. In summary, for the screening process reverse transcription will be performed at 37°C for 90 minutes using M-MLV RT, followed by reverse transcription inactivation at 98°C for 5 minutes. The cycling parameters for PCR that will be used are those described in method 8.

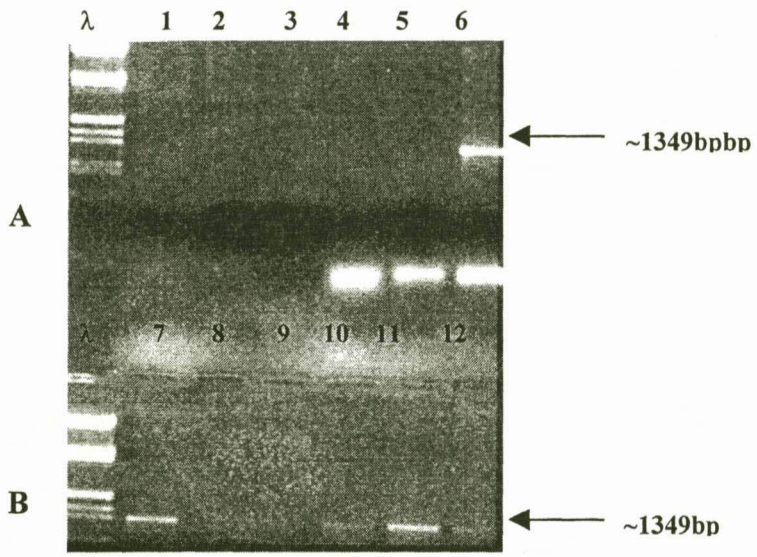


Figure 4.9 Optimisation products of RT-PCR method 8. Mg^{2+} concentration was optimized in two groups of experiments. **Panel A**: Mg^{2+} aliquoted from a 25mM stock solution **Panel B**: optimization was performed in the presence of $MgCl_2$ containing PCR buffer. λ Lambda DNA ladder.

The use of a two step method, although it entails more sample handling, than the one step method, has the advantage of allowing one to be able to target either reverse transcription

or PCR for optimisation. This delineated the problem much quicker than was experienced with the one tube system.

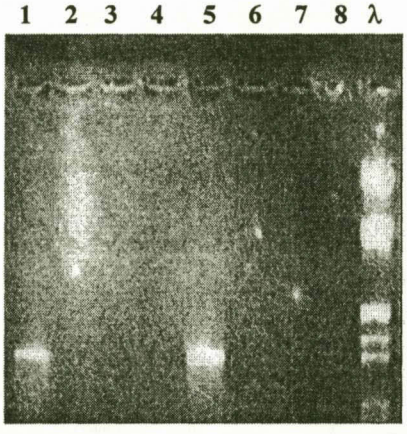
A substantial amount of primer dimers were observed in the optimised reaction that will be applied for the screening process (Figure 4.9, Lane 6). The disadvantage of having primer dimers present in the reaction mixture will necessitate the purification of the band of interest. This will be essential, as the amplimers produced will subsequently be subjected to restriction enzyme analysis. The restriction enzymes profiles produced will be used to group the field strains into the genotypes described by Ballagi-Pordány *et al.*, (1996), the presence of primer dimers will cause confusion with small fragments that will be released during digestion.

Up until this point, substantial work has been performed on the optimisation of this PCR test. Using the above method, consistent results with the control samples of NDV were obtained and will subsequently be used for the screening of the field isolates under investigation.

4.5.3 RT-PCR AMPLIFICATION OF FIELD STRAINS

Amplification of fusion protein gene target sequence

The optimised RT-PCR (method 8) for the amplification of the target sequence on the fusion protein gene using primers ONDV1aa and ONDV4aa was used to screen the field isolates obtained.



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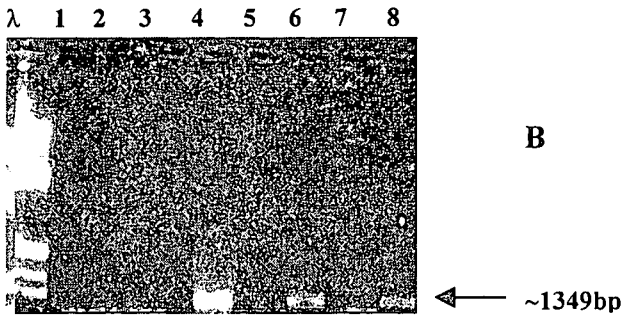

Figure 4.10 RT-PCR Amplification of field strains. RNA extracted (Extraction method 4) from the field strains under investigation was used as template for RT-PCR amplification. M-MLV RT was used for reverse transcription, and Taq polymerase used for amplification. λ: Lambda DNA ladder. **Panel A** Lane1: LaSota, lane 5: M89/98 (Ost Pool 1). **Panel B** Lane 4: M308/98, lane 6:M89/98 (Av. Pool 2), lane 8: M57/98

Four of the 22 field strains obtained gave positive amplification products upon amplification using primers ONDV1aa and ONDV4aa (Figures 4.9 (A), and 4.9 (B)). Three of the four strains gave positive amplification products with the first attempt (Figure 4.10, B). No primer dimers were observed in the amplification of field strains using the optimised reaction mixture of the two step method 8.

After numerous failed attempts at amplifying the same sequence in the rest of the field strains using optimised RNA extraction and RT-PCR conditions, it was decided at this point to perform HA/HI tests. The HA/HI test results confirmed those obtained from RT-PCR amplification that the majority of the strains observed were not NDV. The four strains that gave positive RT-PCR amplimers for the fusion protein gene were investigated further.

Matrix protein gene amplification

A 5μL aliquot of the cDNA mixture used for the amplification of the fusion protein gene sequence were used as template for the PCR amplification of a region comprising 88% of the matrix protein gene.

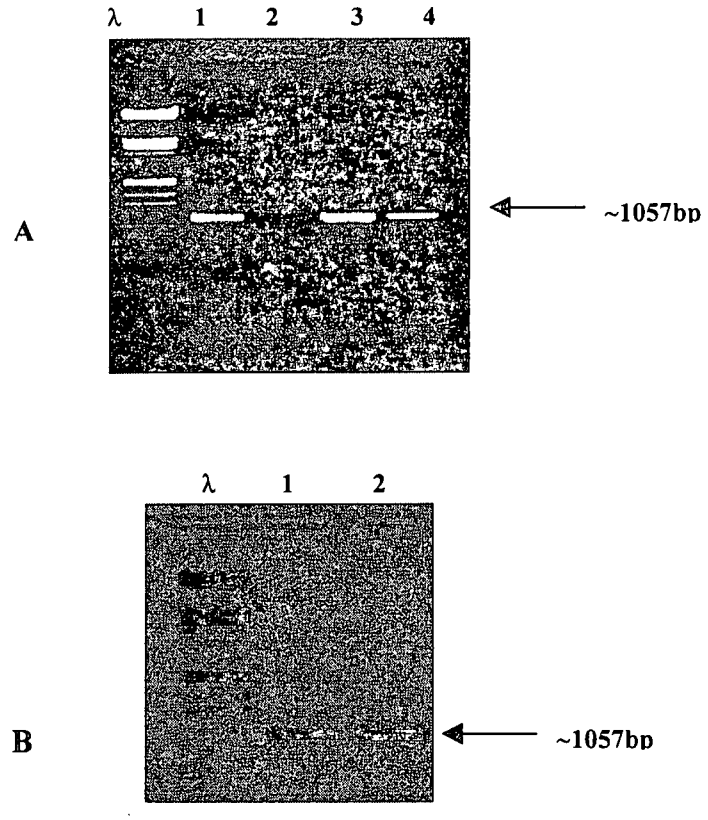

Figure 4.11 RT-PCR amplification of a region of the matrix protein gene. **Panel A**: Lane 1:M89/98 (Ost Pool 1), lane 2: M89/98 (Av. Pool 2), lane 3: M308/98, lane 4: M57/98. **Panel B**: Lane 1: LaSota, lane 2: M89/98 (Av. Pool 2). λ Lambda DNA ladder. The arrows indicate the desired band of ~1057bp

The amplicon of interest was produced with the first attempt (Figure 4.11, panel A) with strains M89/98 (Ost Pool 1), M308/98, and strain M57/98. Primer dimers were observed in all three samples and the band of interest had to be purified. This was achieved using the NucleoSpin Extract Kit. A non-specific amplification product was observed with strain M89/98 (Ost Pool 1). Strain M89/98 (Av. Pool 2) produced a positive amplification signal upon a second attempt at amplification (Figure 4.11, panel B). The amplicons produced were further analysed by RE analysis using MbO I and Hinf I (Chapter 5).

Fusion protein cleavage site amplification

Similarly, as in the amplification of the matrix protein gene sequence, a 5µL cDNA aliquot (optimised method 8), was used as template in the amplification of a sequence within the fusion protein gene that included the fusion protein cleavage site.

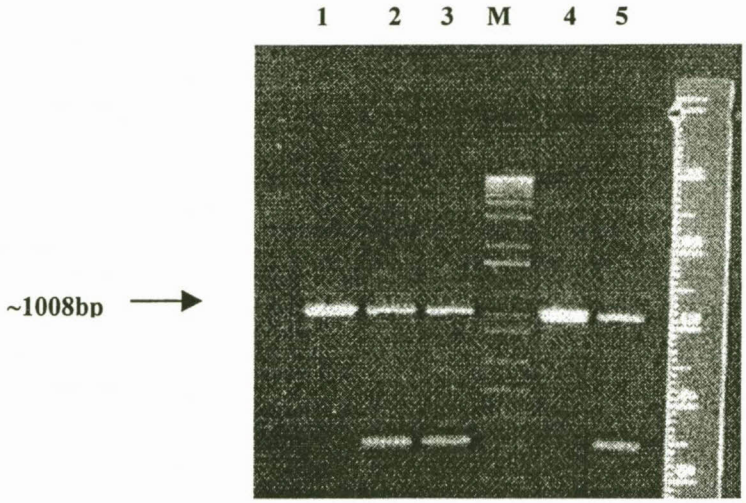

Figure 4.12 RT-PCR products observed after amplification using primers K1 & K2. Lane 1: LaSota, lane 2: M89/98 (Ost Pool 1), lane 3: M89/98 (Av. Pool 2), lane 4: M308/98, lane5: M57/98. M 1Kb Plus DNA ladder.

Amplification of this particular target sequence was achieved by means of a nested PCR. The first PCR was performed using primers K1 and K2 (Figure 4.12). A good yield of the amplification product was obtained, as indicated by the intensity of fluorescence displayed by these bands (Figure 4.12). Amplification products were obtained in reactions of the four field isolates, and also in that of the control strain LaSota. A non-specific amplification product ~100bp was observed with strains M89/98 (Ost Pool 1), M89/98 (Av. Pool 2), and M57/98. This band was not seen with LaSota and M308/98 (Figure 4.12). A PCR coring technique was used to extract the fragment of interest, from these contaminating bands. The amplification products of interest were amplified further in a nested PCR using primes MV1 and B2 (Figure 4.13).

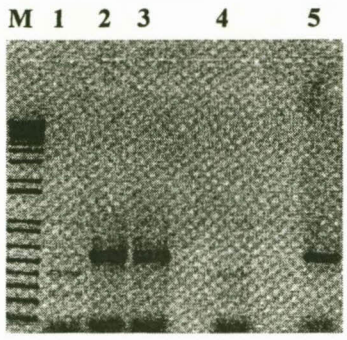

Figure 4.13 Nested PCR amplification products . Lane 1: LaSota, lane 2: M89/98 (Ost Pool 1), lane 3: M89/98 (Av. Pool 2), lane 4: M308/98, lane 5: M57/98. M 1Kb Plus DNA ladder.

Positive amplification results were obtained with strains M89/98 (Ost Pool 1), M89/98 (Av. Pool 2), and M308/98 upon the first attempt of nested PCR. The positive control strain LaSota and field strain 12.1 did not produce amplification products of the desired size (Figure 4.13). These strains produced non-specific amplification products smaller in size than those expected (Figure 4.13 lane 1 & 4).

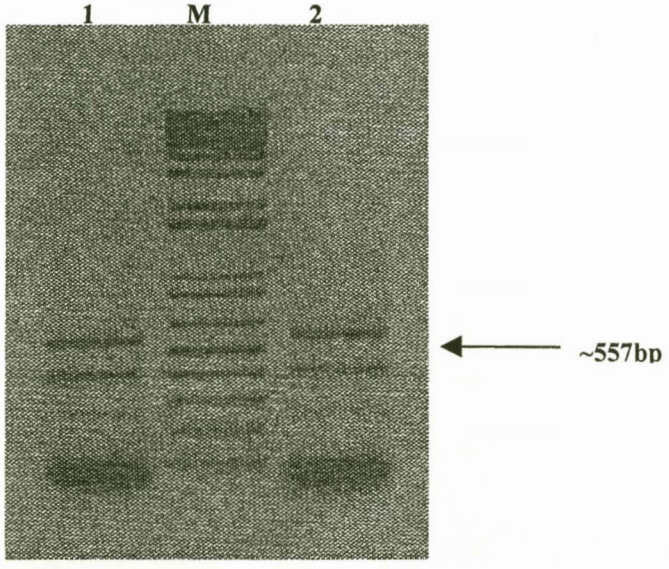

Figure 4.14 Nested PCR amplification products of LaSota and M308/98. Lane 1: LaSota, lane 2: strain M308/98. M 1Kb Plus DNA ladder.

The desired nested PCR products were obtained upon repetition using strains M308/98 and the LaSota control strain (Figure 4.14). Comparing the banding patterns obtained for the other three strains and to that of M308/98, and LaSota it was observed that the latter

two resulted in non-specific products that displayed a similar banding pattern. These bands were not observed with field strains M89/98 (Ost Pool 1), M89/98 (Av. Pool 2), and M57/98.

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CHAPTER 5:

RESTRICTION ENZYME ANALYSIS, SEQUENCING, & PHYLOGENETIC ANALYSIS OF RT-PCR AMPLICONS

5.1 INTRODUCTION

The typing of viruses is an important tool used to characterize viral populations and to study their epidemiology (Charrel & Chandler, 1999). Traditionally, serological methods were used to identify antigenic differences among virus populations (Charrel & Chandler, 1999). With the advent of monoclonal antibody (mAb) technology, it was possible to group NDV isolates into ten antigenic groups that were epizootiologically linked (Alexander *et al.*, 1997).

These techniques mentioned above have been largely replaced by nucleotide or deduced amino acid data that provide more concise, epidemiological information (Charrel & Chandler, 1999). The use of molecular techniques, PCR based methods, pulse field gel electrophoresis and ribotyping are thought to provide data too weak for discriminative typing purposes (Charrel & Chandler, 1999). In contrast, Ballagi-Pordány and co-workers (1996) have applied RT-PCR and restriction site analysis for differentiating and identifying over two hundred NDV strains.

This form of genetic analysis of NDV strains by restriction site mapping of a 75% region of the fusion protein gene, has enabled their grouping into six major groups or genotypes (Ballagi-Pordány *et al.*, 1996). Using this technique, previously separate mAb binding groups were unified into single genotypes (Ballagi-Pordány *et al.*, 1996). Restriction enzyme analysis of an amplicon comprising 88% of the matrix gene provided cleavage maps for reliable and rapid means of differentiating between commercially available vaccine strains LaSota and B-1 (Wehmann *et al.*, 1997).

Phylogenetic analysis based on a 378nt amplicon of the fusion protein gene conformed to those reported for the entire fusion protein gene (Toyoda *et al.*, 1989), and the haemagglutinin neuraminidase gene (Sakaguchi *et al.*, 1989).

NDV strains isolated from epizootics in South Africa and Mozambique (1990/1995) were screened using this RT-PCR based technique. This led to the discovery of two novel groups, VIIb and VIII (Herzceg *et al.*, 1999). Genotype VIII isolates were implied in endemic infections in the region, whilst dendogram data indicate that VIIb isolates are not indigenous to South Africa (Herzceg *et al.*, 1999).

RT-PCR amplicons obtained from the fusion and matrix protein genes of the field strains obtained were subjected to restriction enzyme analysis to determine the genotypes to which they belong. These results were confirmed by sequencing a 378nt region of the fusion protein gene, and subsequent phylogenetic analysis.

5.2 REAGENTS AND CHEMICALS

The reagents and chemicals used were of molecular biological or analytical grade. The chemicals used were obtained from the following companies:
Merck, Germany: EDTA, $CaCl_2$, MgCl2; **Saarchem (Pty) Ltd.:** NaCl, glycerol; **Univar, SA**: tri-sodium citrate, **Boehringer-Mannheim, Germany**: Tris base, **Univar, S.A.**: absolute ethanol, n-butanol **Sigma, USA:** N-Lauroylsarcosine sodium salt, isoamylalcohol, ampicillin, **BioSolve (ltd), Netherlands**: biophenol, IPTG, X-Gal, **Macherey-Nagel, Germany**: NucleoSpin Extraction Kit, **Gibco-BRL, USA**: 1Kb Plus DNA Ladder, **Promega, USA.**: nuclease free ddH_2O, BstO I, Hinf I, Rsa I, MbO 1, BSA, pGEM®-TEasy Vector, **Biolab Diagnostics (Pty) ltd, SA:**, tryptone, yeast extract, **BDH Pty Laboratories, UK:** Triton X-100; **Roche Molecular Biochemicals, SA:** lysozyme, **ABI Prism Applied Biosystems, UK:** BigDye Terminator Cycle Sequencing Kit

5.3 RESTRICTION ENZYME DIGESTION AND SEQUENCING OF RT-PCR AMPLICONS

5.3.1 Restriction enzyme digestion

Concentration of PCR products

Reactants of PCR reactions from tube number 6 (Table 3.3) were concentrated according to the method described by (Herczeg *et al.*, 1999). A 1ml volume of n-butanol was added to a 1.5ml centrifuge tube along with the PCR mixture and vortexed for 15 seconds. The DNA was pelleted by centrifugation (12 X 1500g for 10minutes at 4°C) and the supernatant discarded. The pellet was dried under vacuum and finally re-dissolved in 18μL twice distilled (dd) nuclease free H_2O (NFW).

RE digestion of F protein amplicons

The 18μL concentrated PCR products were divided into three equal fractions of 5μL each in sterile eppendorf tubes. Subsequently, the DNA was digested in 20μL reaction containing 5U of the restriction enzymes *BstO O1*, Hinf 1, and Rsa 1 respectively. Digestion was performed in the presence of 0.1μg/μL BSA at 37°C for *Hinf* 1, and *Rsa* 1, and at 50°C for *BstO* O1 for at least four hours, according to the manufacturers' recommendations.

Each of the above reaction mixtures was mixed with 5μL loading buffer and analysed by electrophoresis. A 1Kb Plus DNA ladder was included to estimate the size of the resultant fragments. Separation of the fragments was facilitated by electrophoresis (185V for 2hrs) on a 2.5% agarose gel stained in ethidium bromide (1.5mg/ml) and visualized in a UV-transilluminator (Herczeg *et al.*, 1999).

RE digestion of M protein amplicons

M-protein gene amplicons were analysed on an ethidium bromide stained, 1% agarose gel. Reactions containing the amplimers of interest were concentrated using n-butanol as described previously. Purification of PCR products of interest was performed as previously described (NucleoSpin Extraction Kit).

RE analysis was performed using restriction enzymes Hinf 1, and MbO 1 in two separate reactions of 10µL each. For digestion with MbO 1 reactants were added to final concentrations of 1 X RE buffers (C), 2µg/µL BSA, 5U *MbO* 1, 5µL PCR product and made up to 10µL with NFW.

RE analysis with Hinf 1 was performed in a 10µL reaction volume containing reaction components at final concentrations of 1 X RE buffer (B), 2.0µg/µL BSA, 5U Hinf 1, and 5µL PCR product and made up to a total volume of 10µL with NFW. Digestion was allowed to proceed at 37°C for 6 hours. The entire reaction volume was loaded on a 1 % (w/v) ethidium bromide stained agarose gel and analysed by UV-transillumination.

5.3.2 Sequencing of the fusion protein cleavage site amplicons

Cloning of RT-PCR amplicons

Preparation of competent *E. coli* Top 10 Cells

To facilitate sequencing PCR fragments of the nested PCR reaction were cloned into pGEM®-TEasy vectors. Cloning into this vector facilitated the sequencing of the 5` and 3` flanking regions of the PCR products using primers Sp6 and T.

E. coli Top 10 cells were precultured in 100ml LB medium (10g Bacto®-tryptone , 5g Bacto®-Yeas extract , 5g NaCl) overnight at 37°C with shaking. Subsequently a 10^{-2} aliquot of the preculture was inoculated into 5ml LB media and incubated at 37°C with

shaking. Optical densities at 600nm, were monitored until an OD of 0.9-0.95 was reached, after which the cells were pelleted (5 X 1000g for 5 minutes at 4°C) using a pre-cooled rotor. The cell pellets were resuspended in a 10ml ice-cold solution of 80mM $CaCl_2$ and 50mM $MgCl_2$ and incubated for 10 minutes on ice. The above treatment was repeated twice. Finally, the pellet was resuspended in a 2ml ice-cold solution of 0.1mM $CaCl_2$ and mixed with an equal volume of 50% glycerol. The cells were aliquoted into 80µL fractions in 1.5ml eppendorf tubes, snap-frozen in liquid nitrogen, and stored at -70°C (Tang *et al.*, 1994)

Ligation into pGEM®-T Easy Vectors

The pGEM®-T Easy vector was collected by brief centrifugation (Eppendorf centrifuge 5417R, Germany). The ligation mixtures were assembled in 0.5ml centrifuge tubes of low-DNA-binding capacity (Table 5.1)

Table 5.1 Final concentrations of reaction components assembled for ligation of Nested PCR amplicons into pGEM®-T Easy Vectors.

Reagent	Concentration
2 X Rapid Ligation Buffer	1 X
pGEM®-TEasy Vector (50ng)	2.5ng/µL
PCR product	3.5µL
T_4 DNA Ligase 3U/µL	1.0µL
Total Volume	10µL

The reagents were mixed gently by pipetting, and ligation allowed to proceed at 4°C overnight.

Transformation into competent *E. Coli* Top 10 cells

When the cells were required for transformation, they were thawed on ice and an aliquot of 2-10µL of DNA (nested PCR amplicons) was added to the cell suspension. A negative

control reaction that did not contain any insert was prepared. Subsequent to mixing, the reaction mixture was incubated on ice for exactly 30 minutes.

Heat shock was carried out at 42°C for 90 seconds, followed by incubation on ice for 2 minutes (Tang *et al.*, 1994). Subsequently 400µL LB medium and 40mM glucose were added, followed by incubation at 37°C for one hour with shaking. The transformants were pelleted for 40 seconds at 14 x 1000g at room temperature. The supernatant fluid was removed, and the cells resuspended in the remaining 100µL. The transformation cultures were then plated onto LB plates containing 100µg/µL ampicillin, 0.5mM IPTG, and 80µg/ml X-Gal. This would readily allow the selection of transformants through blue/white colony selection (pGEM®-T and pGEM®-TEasy Vector Systems, Technical manual, Promega, 1999). Clones that contained the insert of interest produced white colonies. These were isolated using sterile Gilson p10 white tips, and inoculated into 5ml LB containing 0.1mg/ml ampicillin. The cells were incubated at 37°C with shaking for 24 hours.

Small scale isolation of plasmid DNA

Insert containing plasmids were isolated by pelleting the transformants by centrifugation (14 x 1000g at room temperature for 1 minute). The cell pellets were resuspended in STET buffer pH (8.0) (100mM NaCl, 5%(v/v) Triton X-100, 10mM Tris-HCl pH 8.0, 1mM EDTA), and a 4µL aliquot of lysozyme (50mg/ml) added. The cells were lysed by incubating the sample for 60 seconds in a boiling waterbath. The cell suspension was placed on ice for 10 minutes and the debris collected by centrifugation (15 minutes at 14 X 1000g at room temperature). Subsequently the resulting pellet was extracted using a sterile toothpick. Plasmid DNA was isolated by the addition of 200µL of isopropanol and incubation at -20°C for 10 minutes. The plasmids were pelleted by centrifugation (14 x 1000g for 10 minutes), the supernatant discarded and plasmid DNA dried under vacuum in a rotatory evaporator. The DNA was resuspended in 50µL aliquots of TE buffer containing 100µg/µL RNase. The DNA was analysed by electrophoresis on a 1%

(w/v) agarose gel containing ethidium bromide (0.5μg/μL) in TE buffer pH 8/0 (Adapted from Sambrook *et al.*, 1989).

RE digestion of Plasmid DNA

The clones isolated above were analysed for the presence of the insert of interest by restriction enzyme analysis. The vector used, pGEM®-TEasy, allows for the release of an insert by single-enzyme digestion using any of the enzymes EcoR I, Bstz I, and Not I (pGEM®-T and pGEM®-TEasy Vector Systems, Technical Manual, Promega, 1999).

Plasmid DNA was digested with EcoR I according to the suppliers' recommendations, generally 3μL of plasmid DNA was digested in a 10μL reaction volume containing 1U of restriction enzyme. Digestion was allowed to proceed at 37°C overnight.

Restriction fragments were analysed by electrophoresis through a 1% (w/v) agarose gel stained in ethidium bromide in TE buffer. Plasmid preparations found to contain the insert of interest were purified further.

Plasmid DNA Purification

Briefly, the bacterial cells were pelleted by centrifugation (14 X 1000g for 1 minutes), and the pellet resuspended in 250μL buffer A1. Cell lysis was performed by the addition of 250μL of buffer A2, and incubation at room temperature for exactly 5 minutes. Thereafter 300μL of buffer A3 was added to the suspension, and mixed by inversion, followed by incubation on ice for 5 minutes. The bacterial debris was pelleted by centrifugation (12 X 1000g for 12 minutes at 4°C). The supernatant fluid was loaded onto a NucleoSpin column placed in a 2μL centrifuge tube. This was followed by centrifugation (60 seconds at 14 X 1000g), and the flow-through discarded. A 700μL aliquot of buffer A4 was used to wash the NucleoSpin column (centrifugation at 14 x 1000g for 60 seconds). The flow-through was discarded and centrifugation repeated to remove residual ethanol. The NucleoSpin column was placed in a new centrifuge tube ,

and the DNA eluted by the addition of 50μL elution buffer TE and centrifugation (60 seconds at 14 X 1000g).

Sequencing of cloned inserts

Clones containing the inserts of interest were subjected to sequencing using the BigDye Terminator Cycle Sequencing Kit according to the manufacturers instructions.

Sequencing was performed in 20μL reactions of 300ng DNA, 3.2pmol of each of the primers T7 and Sp6, 8μL of the Terminator ready reaction mix. The reagents were mixed well and collected by brief centrifugation. The reagent containing tubes were placed in a GeneAmp PCR System 2400 (Perkin Elmer) set at 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. This was followed by a rapid thermal ramp to 4°C and maintained at this temperature until further purification.

Precipitation of cloned fragments

Once the samples were ready for purification, the contents were collected by centrifugation (1 X 1000g for 1minute), and transferred to a 1.5ml centrifuge tube into which aliquots of 2μL of 3M NaOAC and 50μL of 95% EtOH were mixed. The mixture was incubated at room temperature for 15 minutes after which the DNA was pelleted (14 X 1000g for 20 minutes at 4°C), and the supernatant discarded. The pellet was washed by the addition of 250μL of 70% ethanol and centrifuged at (14 X 1000g for 5minutes at 4°C). Once the supernatant was discarded, the pellet was dried under vacuum and resuspended in 5μL formamide loading dye, containing 25mM EDTA. Subsequently, denaturation was performed at 95°C for 2 minutes, and the DNA sequenced on a 4% acrylamide gel. The programs used in the procedure were all Perkin Elmer products that were run on an Apple Macintosh Power PC. The data was collected on an ABI Prism 377 DNA Sequencer (Perkin Elmer). Analysis was performed using Sequencing Analysis 3.3. The following programs were used to analyse the sequences, Sequence Navigator V 1.0.1 was used to compare reverse complemented sequences, and the sequences assembled using AutoAssembler V 1.4.0.

5.4 RESULTS AND DISCUSSION

5.4.1 RE profiles of fusion protein gene amplimers

RT-PCR amplimers generated using primers ONDV1aa and ONDV4aa were subjected to RE digestion using three enzymes, Hinf I, BstO I, and Rsa I (Ballagi-Pordány *et al.*, 1996).

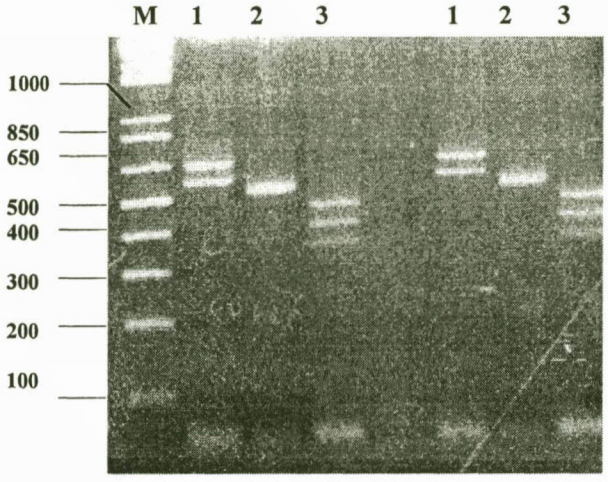

Figure 5.1 RE profile of LaSota F gene amplicon. Restriction enzyme profile of amplicons representing a 75% region of the fusion protein gene of NDV strain LaSota. This experiment was performed in two separate reactions. Lane 1: BstO I digestion profile, lane 2: Hinf 1digestion profile, lane 3: Rsa 1 digestion profile, M (1Kb Plus DNA Ladder)

This commercially available vaccine strain (LaSota) was used as a positive control strain throughout this study. The restriction enzyme profiles produced from the digestion of its amplimers will be used to detect if any of the field strains used in the study were re-isolated forms of this virus, since it is a commonly used live vaccine strain in the region.

This strain belongs to genotype II, as described by Ballagi-Pordány *et al.,* 1996. Digestion of this amplimer with BstO I, resulted in fragments of the following sizes, ~646bp, ~567bp, ~137bp. *Hinf* 1 digestion profile consisted of fragments of ~541bp, and ~525bp (Figure 5.1). The *Rsa* I digested amplimer produced bands of ~349bp, ~404bp, and 465bp in size (Figure 5.1).

These are similar to those previously reported for this particular strain.

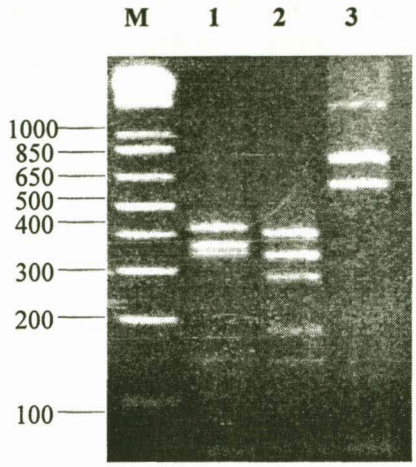

Figure 5.2 Restriction fragment pattern of PCR amplicon of NDV field strain M89/98 (Ost Pool 1). The digested fragments were separated on a 1% agarose gel and stained in ethidium bromide. Lane 1: BstO I digests, lane 2: Hinf I digests, lane 3: Rsa I digests. M (1Kb Plus DNA Ladder, GibcoBRL).

Digestion of the amplimer derived from strain M89/98 (Ost Pool 1) with BstO 1 produced fragments of the following sizes: ~419bp, ~364bp, ~141bp (Figure 5.2 (A), lane 1). A 81bp fragment which should migrate below the 100bp marker band was not clearly visible. *Hinf* 1 digestion released the fragments observed in this group i.e., ~401bp, ~336bp, ~282bp, and ~181bp, and ~139bp (Figure 5.2, lane 2). The banding pattern produced with Rsa I digestion released fragments ~754bp and ~595bp in size (Figure 5.2 (A), lane 3). A larger fragment, ~1349bp in size, observed in lane 3, is the undigested RT-PCR amplimer.

The restriction pattern from digestion of the fusion protein amplimer of field strain M89/98 (Ost Pool 1) is different from that of the LaSota control strain. The pattern produced by this strain is similar to that of strains belonging to genotype VIIb (Herczeg *et al.*, 1999).

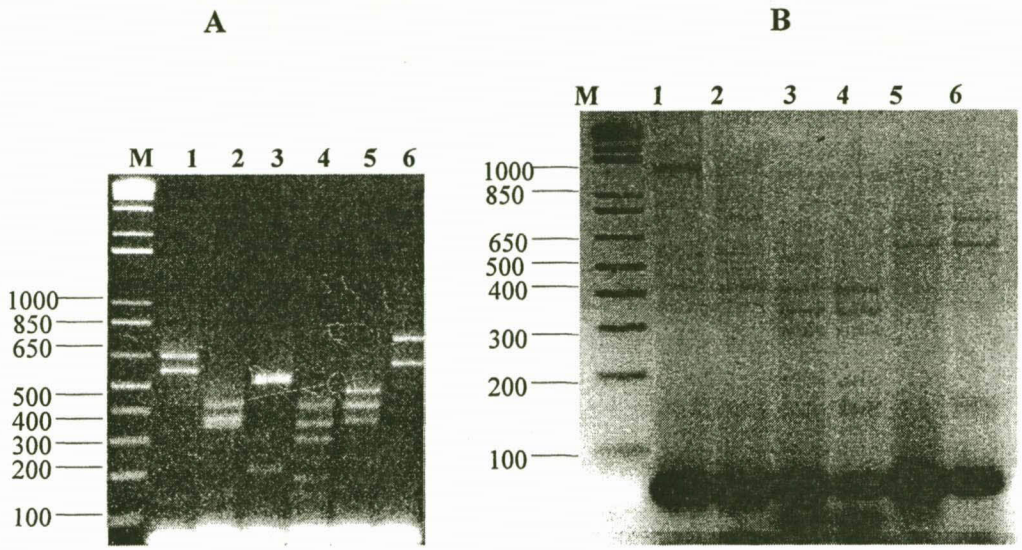

Figure 5.3 Restriction fragment pattern of PCR amplicon of NDV field strains M89/98 (Av. Pool 2), M57/87, and M308/98. The digested fragments were separated on a 1% agarose gel and stained in ethidium bromide. **Panel A**: Lane 1 & 6: strains M308/98 and M57/98 amplimers digested with *Rsa* 1 respectively, lanes 5 & 2: strains M308/98 and M57/98 amplimers digested with *BstO O1* respectively, lanes 3 & 4: strains M308/98 and M57/98 amplimers digested with *Hinf* 1 respectively. **Panel B** : Lanes 1 & 2: strains M89/98 (Av. Pool 2) and M308/98 amplimers digested with *BstO O1* respectively, lanes 3 & 4: strains M89/98 (Av. Pool 2) and M308/98 amplimers digested with *Hinf* 1, lanes 5 & 6: strains M89/98 (Av. Pool 2) and M308/98 amplimers digested with *Rsa* 1, M (1Kb Plus DNA Ladder, GibcoBRL).

The restriction enzyme pattern of the fusion protein amplimer of field strain M57/98 upon digestion with *BstO O1* includes all the fragments mentioned for strain M89/98 (Ost Pool 1) (Figure 5.3 (A), lane 2). The smallest fragment of 81bp is not visible. The fragment patterns produced from digestion with *Rsa* 1 (Figure 5.3 (A), lane 6), and *Hinf* 1 (Figure 5.3 (A), lane 4) are identical to those of isolates of genotype VIIb.

Rsa 1 digestion of strain M308/98 RT-PCR amplimer only released a fragment of ~595bp that is characteristic of genotype VIIb strains (Figure 5.3 (A) lane 1). A smaller fragment of ~540bp in size was observed. When this strain was digested with *BstO O1* (Figure 5.3 (A), lane 5) two fragments seen in genotype VIIb were visualized (~401bp & ~336bp). A larger fragment of ~500bp was observed, whilst it lacked a fragment of ~282bp.

The restriction enzyme pattern produced by the digestion of strain M308/98 with *Hinf* 1 displayed fragments of the following sizes: ~517bp, ~519bp, and ~230bp (Figure 5.3 (A) lane 3). This banding pattern is not consistent with that observed for genotype VIIb isolates. Fragments 517 and 519 have been observed among genotype II strains (Ballagi-Pordány, 1996).

Restriction fragment patterns observed from the digestion of RT-PCR amplicons of strains M89/98 (Av. Pool 2) and M308/98 resulted in fragments ~419bp, ~364bp and ~144bp, upon digestion with *BstO O1*(Figure 5.3 (B), lanes 1& 2). This banding pattern contained bands characteristic of genotype VIIb viruses (Herczeg *et al.*, 1999). Fragments larger in size than those reported for group VIIb were observed (Figure 5.3. (B), lanes 1 & 2). Undigested RT-PCR amplicon was also visible. These strains lacked a fragment of ~282bp (Figure 5.3, (B), lanes 1 & 2).

The banding pattern observed upon digestion with *Hinf* 1 (Figure 5.3 (B), lanes 3 & 4) produced bands of the expected size for group VIIb isolates. A fragment of size ~500bp was observed for strain M89/98. The digestion of the fusion protein amplimer of these strains with *Rsa* 1 resulted in bands of the expected sizes i.e., ~754bp, and ~595bp (Figure 5.3 (B), lanes 5 & 6) for both strains. A smaller fragment of ~160bp was observed for strain M308/98.

5.4.2 RE profiles of matrix protein gene amplicons

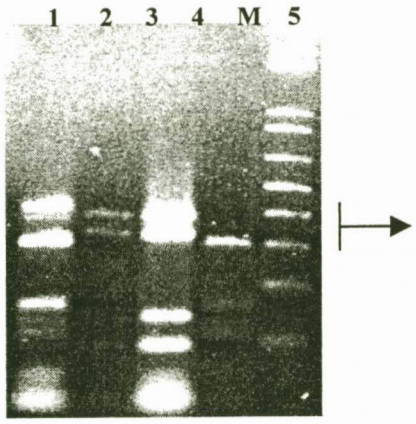

Figure 5.4 Restriction enzyme pattern of the matrix gene amplimers. The 1097 RT-PCR product of field strains and of the control strain LaSota were digested with *Hinf* 1. Lane 1: LaSota, lane 2: M89/98 (Ost Pool 1), lane 3: M89/98 (Av. Pool 2), lane 4: M308/98, lane 4: M57/98, M (1Kb Plus DNA Ladder).

Restriction fragments of strains M89/98 (Ost Pool 1), M89/98 (Av. Pool 2) and M57/98 (Figure 5.4), displayed two fragments of ~320bp and ~370bp in size. Strain M57/98 contained the smaller of the two.

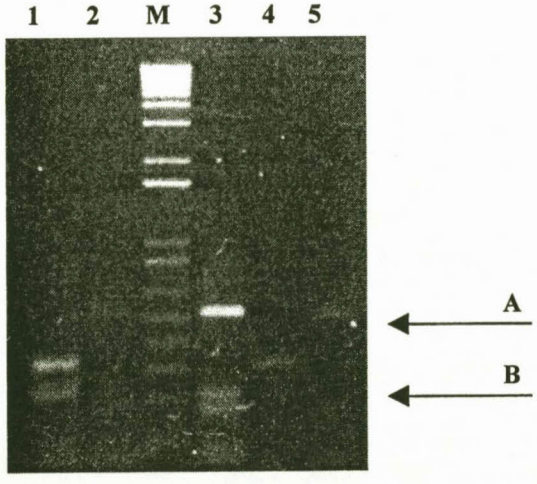

Figure 5.5 Restriction enzyme pattern of the matrix gene amplimers. The 1097 RT-PCR product of field strains and of the control strain LaSota were digested with MbO 1. Lane 1: LaSota, lane 2: M89/98 (Ost Pool 1), lane 3: M89/98 (Av. Pool 2), lane 4: M308/98, lane 4: M57/98, M (1Kb Plus DNA Ladder).

The banding pattern observed for strains M89/98 (Ost Pool 1), M89/98 (Av. Pool 2) and M57/98 had a number of bands in common: a ~500bp (Arrow A), and a smaller band of~180bp in size (Arrow B). The banding pattern of strain M308/98 was different from the three mentioned above, as it lacked the ~500bp fragment. Strains M89/98 (Av. Pool 2) and M57/98 had a~220bp fragment in common. Overall, different banding patterns were observed between the individual strains (Figure 5.5)

The LaSota banding profile differed from that observed for the field strains. The following fragments were observed in field strains M308/98 and LaSota, ~310bp and ~210bp. The latter of the two fragments was observed in M89/98 (Av. Pool 2).

5.4.3 Sequencing of the fusion protein cleavage site amplicons.

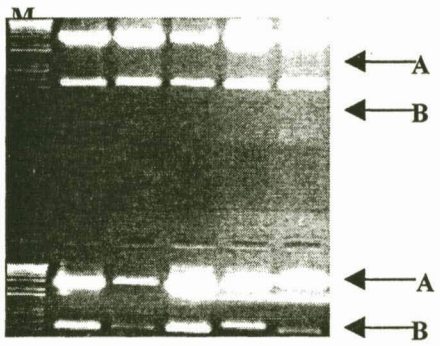

Figure 5.6 Restriction enzyme analysis using EcoR 1. Plasmid was extracted from clones that showed positive transformation. The plasmid preparations were subjected to EcoR I digestion in order to confirm that the clones contained fragments of the expected size. M: Lambda DNA ladder. Arrow A: Plasmid DNA. Arrow B: RT-PCR fragments.

RT-PCR products 378nt in length were cloned into pGEM®-TEasy vectors and transformed into *E.coli*. Positive transformants were isolated; the vector extracted and subjected to EcoR 1 digestion. Two clones were selected for each sample. All the clones were found to contain the fragment of interest (Figure 5.6, Arrow B). Arrow A (Figure 5.6) indicates the plasmid backbone.

Double stranded DNA Sequences of cloned RT-PCR fragments

```
NS 6B  1   GAATTCGATTGCTGGATAATGATCTATCTTGATTGTCTGTAGTTAGTTCACCTTGTCTATCTTAATT 67
NS 3B  1   GAATTCGATTGCTGGATAATGATCTATCTTGATTGTCTGTAGTTAGTTCACCTTGTCTATCTTAATT 67

NS 6B  68  AGAAAAAACACGGGGTAGAAGAGTCTGGATCCCGACTAGCACATTCAAAACGTAATATGGGCTCCAA 134
NS 3B  68  AGAAAAAACACGGGGTAGAAGAGTCTGGATCCCGACTAGCACATTCAAAACGTAATATGGGCTCCAA 134

NS 6B  135 ACCCTCTACCAGAATCCCAGTACCCCTGATGCTGATCACTCGGGTTATGCTGATCTTAAGCTGTATC 201
NS 3B  135 ACCCTCTACCAGAATCCCAGTACCCCTGATGCTGATCACTCGGGTTATGCTGATCTTAAGCTGTATC 201

NS 6B  202 TGTTCGACAGGTTCCCTTGATGGCAGGCCTCTTGCAGCTGCAGGGATTGTAGTGACAGGAGATAAGG 268
NS 3B  202 TGTTCGACAGGTTCCCTTGATGGCAGGCCTCTTGCAGCTGCAGGGATTGTAGTGACAGGAGATAAGG 268

NS 6B  269 CAGTCAATATATACACCTCATCTCAGACTGGGTCAATCATAGTCAAATTGCTCCCGAATATGCCCAA 335
NS 3B  269 CAGTCAATATATACACCTCATCTCAGACTGGGTCAATCATAGTCAAATTGCTCCCGAATATGCCCAA 335

NS 6B  336 GGATAAAGAGGCGTGTGCAAAAGCCCCATTAGAGGCATACAACAGAACACTTGACCACCTTACTCAC 402
NS 3B  336 GGATAAAGAGGCGTGTGCAAAAGCCCCATTAGAGGCATACAACAGAACACTTGACCACCTTACTCAC 402

NS 6B  403 TCCCCTTGGCGATTCTATCCGTAGGATACAAGGGTCTGTGTCCACATCGGGAGGAAGGAGACAGAAA 469
NS 3B  403 TCCCCTTGGCGATTCTATCCGTAGGATACAAGGGTCTGTGTCCACATCGGGAGGAAGGAGACAGAAA 469

NS 6B  470 CGCTTCATAGGTGCCCGTTATTTGGCAAGTGTAGCTCTTGGGGGTTGCAACAGCGGCACAGATAACA 536
NS 3B  470 CGCTTCATAGGTGCCCGTTATTTGGCAAGTGTAGCTCTTGGGGGTTGCAACAGCGGCACAGATAACA 536

NS 6B  537 GCAGCTTGCGGCTCTAATACCAGCCAAACAGAATGCTGC 575
NS 3B  537 GCAGCTTGCGGCTCTAATACCAGCCAAACAGAATGCTGC 575
```

Figure 5.7 Double stranded DNA sequence of strain (M57/98). RT-PCR fragments were sequenced in both directions. The fusion protein cleavage site is indicated in bold face

```
NS2B   1   GAATTCACTAGTGATTACTGGATAATGATCTATCTTGATTGTCTGTAGTTAGTTCACCTGTCTATCT 67
NS 5B  1   GAATTCACTAGTGATTACTGGATAATGATCTATCTTGATTGTCTGTAGTTAGTTCACCTGTCTATCT 67

NS2B   68  AATTAGAAAAAACACGGGTAGAAGAGTCTGGATCCCGACTAGCACATTCAAAACGTAATATGGGCTC 134
NS 5B  68  AATTAGAAAAAACACGGGTAGAAGAGTCTGGATCCCGACTAGCACATTCAAAACGTAATATGGGCTC 134

NS2B   135 CAAACCTTCTACCAAGAATCCCAGTACCCCTGATGTTGATCACTCGGGTTATGCTGATCTTAAGCTG 201
NS 5B  135 CAAACCTTCTACCAAGAATCCCAGTACCCCTGATGTTGATCACTCGGGTTATGCTGATCTTAAGCTG 201

NS2B   202 TATCTGTTCGACAGGTTCCCTTGATGGCAGGCCTCTTGCAGCTGCAGGGATTGTAGTGACAGGAGAT 268
NS 5B  202 TATCTGTTCGACAGGTTCCCTTGATGGCAGGCCTCTTGCAGCTGCAGGGATTGTAGTGACAGGAGAT 268

NS2B   269 AAGGCAGTCAATATATACACCTCATCTCAGACTGGGTCAATCATAGTCAAATTGCTTCCGAATATGC 335
NS 5B  269 AAGGCAGTCAATATATACACCTCATCTCAGACTGGGTCAATCATAGTCAAATTGCTTCCGAATATGC 335

NS2B   336 CCAAGGATAAAGAGGCGTGTGCAAAAGCCCCCTTAGAGGCATACAACAGGACACTGACCACCTTACT 402
NS 5B  336 CCAAGGATAAAGAGGCGTGTGCAAAAGCCCCCTTAGAGGCATACAACAGGACACTGACCACCTTACT 402

NS2B   403 CACTCCCCTTGGCGACTCTATCCGTAGGATACAAAGGGTCTGTGTCCACATCGGGAGGAAGGAGACA 469
NS 5B  403 CACTCCCCTTGGCGACTCTATCCGTAGGATACAAAGGGTCTGTGTCCACATCGGGAGGAAGGAGACA 469

NS2B   470 GAAACGCTTCATAGGTGCCGTTATTTGGCAAGTGTAGCTCTTTGGGGTTGCAACAGCGGCACAGATA 536
NS 5B  470 GAAACGCTTCATAGGTGCCGTTATTTGGCAAGTGTAGCTCTTTGGGGTTGCAACAGCGGCACAGATA 536

NS2B   537 ACAGCAGCTGCGGGCTCTAATACAAGCCAACCAAAATGCTGAATCGAATTC 587
NS 5B  537 ACAGCAGCTGCGGGCTCTAATACAAGCCAACCAAAATGCTGAATCGAATTC 587
```

Figure 5.8 Double stranded DNA sequence of strain (M89/98, Ostr. Pool 1). The 378nt RT-PCR fragment was sequenced in both directions using primers Sp6 and T7. The fusion protein cleavage site is indicated in bold face

```
6       1   GAATTCCTGAATACACTATGACGCTGGATAATGATCTATCTTGATTGTCTGCAGAATGAGTTCACCT 67
6 [1]   1   GAATTCCTGAATACACTATGACGCTGGATAATGATCTATCTTGATTGTCTGCAGAATGAGTTCACCT 67

6       68  GTCTATCTAAATTAGAAAAAACACGGGTAGAAGATTCTGGATCCCGGTTGGCGCCCTCCAGGTGCAA 134
6 [1]   68  GTCTATCTAAATTAGAAAAAACACGGGTAGAAGATTCTGGATCCCGGTTGGCGCCCTCCAGGTGCAA 134

6       135 GATGGGCTCCAGACCTTCTACCAAGAACCCAGCACCTATGATGCTGACTATCCGGGTTGCGCTGGTA 201
6 [1]   135 GATGGGCTCCAGACCTTCTACCAAGAACCCAGCACCTATGATGCTGACTATCCGGGTTGCGCTGGTA 201

6       202 CTGAGTTGCATCTGTCCGGCAAACTCCATTGATGGCAGGCCTCTTGCAGCTGCAGGAATTGTGGTTA 268
6 [1]   202 CTGAGTTGCATCTGTCCGGCAAACTCCATTGATGGCAGGCCTCTTGCAGCTGCAGGAATTGTGGTTA 268

6       269 CAGGAGACAAAGCCGTCAACATATACACCTCATCCCAGACAGGATCAATCATAGTTAAGCTCCTCCC 335
6 [1]   269 CAGGAGACAAAGCCGTCAACATATACACCTCATCCCAGACAGGATCAATCATAGTTAAGCTCCTCCC 335

6       336 GAATCTGCCCAAGGATAAGGAGGCATGTGCGAAAGCCCCCTTGGATGCATACAACAGGACATTGACC 402
6 [1]   336 GAATCTGCCCAAGGATAAGGAGGCATGTGCGAAAGCCCCCTTGGATGCATACAACAGGACATTGACC 402

6       403 ACTTTGCTCACCCCCCTTGGTGACTCTATCCGTAGGATACAAGAGTCTGTGACTACATCTGGAGGGG 469
6 [1]   403 ACTTTGCTCACCCCCCTTGGTGACTCTATCCGTAGGATACAAGAGTCTGTGACTACATCTGGAGGGG 469

6       470 GGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTGGCTCTTGGGGTTGCAACTGCCGCAC 536
6 [1]   470 GGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTGGCTCTTGGGGTTGCAACTGCCGCAC 536

6       537 AAATAACAAGCGGCCGCAAGCTCCAATTCGAATTC 571
6 [1]   537 AAATAACAAGCGGCCGCAAGCTCCAATTCGAATTC 571
```

Figure 5.9 Double stranded DNA sequence of strain (M89/98, Av. Pool 2). The 378nt RT-PCR fragment was sequenced in both directions using primers Sp6 and T7. The fusion protein cleavage site is indicated in bold face

```
12      1   GTATGGGCCCTCGATCGCTGCATTCCGCCGCCATGGCGGCCGGGGAATCATCCCGATCATTGTGAC 66
12 [1]  1   GTATGGGCCCTCGATCGCTGCATTCCGCCGCCATGGCGGCCGGGGAATCATCCCGATCATTGTGAC 66

12      67  ACTAGATAAATGATCTATCTTGATTGTCTGTAAGTTAGTTTACACCTGTCTATCTAATTGAAAAAA 132
12 [1]  67  ACTAGATAAATGATCTATCTTGATTGTCTGTAAGTTAGTTTACACCTGTCTATCTAATTGAAAAAA 132

12      133 AACACGGGTAGAAGAAGTCTGGATCCCGACTAGCACATTCAAAACGTAATATGGGCTCCAAACCTT 198
12 [1]  133 AACACGGGTAGAAGAAGTCTGGATCCCGACTAGCACATTCAAAACGTAATATGGGCTCCAAACCTT 198

12      199 CTACCAGAATCCCAGTACCCCTGATGTTGATCACTCGGGGTTATGCTGATCTTAAGCTGTATCTGT 264
12 [1]  199 CTACCAGAATCCCAGTACCCCTGATGTTGATCACTCGGGGTTATGCTGATCTTAAGCTGTATCTGT 264

12      265 TCGACAGGTTCCCTTGATGGCAGGCCTCTTGCAGCTGCAGGGATTGTAGTGACAGGAGATAAGGCA 330
12 [1]  265 TCGACAGGTTCCCTTGATGGCAGGCCTCTTGCAGCTGCAGGGATTGTAGTGACAGGAGATAAGGCA 330

12      331 GTCAATATATACACCTCATCTCAGACTGGGTCAATCGTAGTCAAATTGCTTTTCCGAATATGCCCA 396
12 [1]  331 GTCAATATATACACCTCATCTCAGACTGGGTCAATCGTAGTCAAATTGCTTTTCCGAATATGCCCA 396

12      397 AGGATAAAGAGGGCGTGTGCAAAAGCCCCCTTAGAGGCATACAACAGAACACTGACCACCTTACTC 462
12 [1]  397 AGGATAAAGAGGGCGTGTGCAAAAGCCCCCTTAGAGGCATACAACAGAACACTGACCACCTTACTC 462

12      463 ACTCCCCTTGGCGACTCTATCCGTAGGATACAAGGGTCTGTGTCCACATCGGGAGGAAGGAGACAG 528
12 [1]  463 ACTCCCCTTGGCGACTCTATCCGTAGGATACAAGGGTCTGTGTCCACATCGGGAGGAAGGAGACAG 528

12      529 AAACGCTTCATAGGTGCCGTTATTGGCAGTGTAGCTCTTGGGGTTGCAACAGCGGCACAAATAACA 594
12 [1]  529 AAACGCTTCATAGGTGCCGTTATTGGCAGTGTAGCTCTTGGGGTTGCAACAGCGGCACAAATAACA 594

12      595 GCAGCTGCGGCTTTATACAA 614
12 [1]  595 GCAGCTGCGGCTTTATACAA 614
```

Figure 5.10 Double stranded DNA sequence of strain (M308/98). The 378nt RT-PCR fragment was sequenced in both directions using primers Sp6 and T7. The fusion protein cleavage site is indicated in bold face

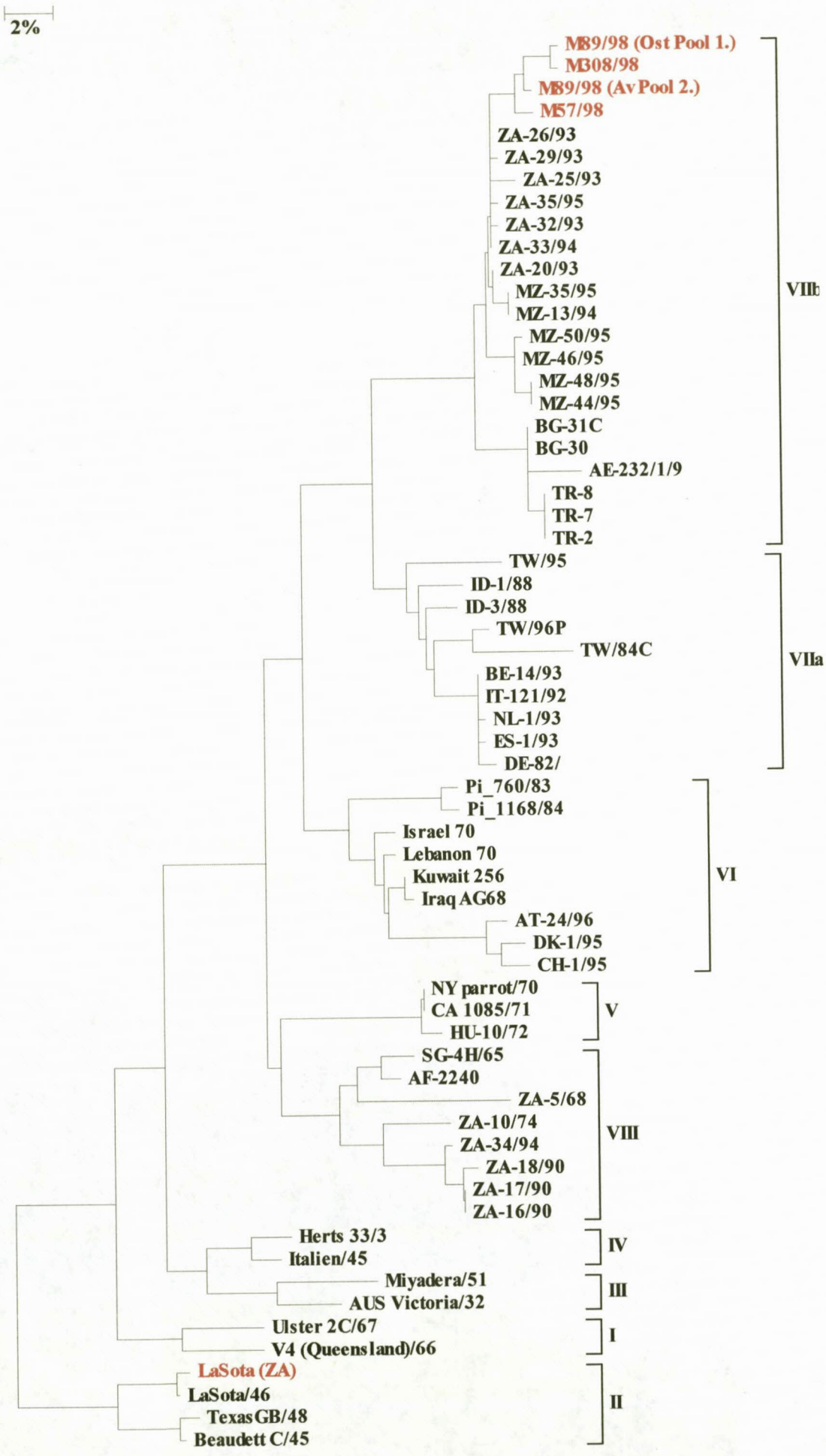
2%
M89/98 (Ost Pool 1.)
M308/98
M89/98 (Av Pool 2.)
M57/98
ZA-26/93
ZA-29/93
ZA-25/93
ZA-35/95
ZA-32/93
ZA-33/94
ZA-20/93
MZ-35/95
MZ-13/94
MZ-50/95
MZ-46/95
MZ-48/95
MZ-44/95
BG-31C
BG-30
AE-232/1/9
TR-8
TR-7
TR-2
VIIb
TW/95
ID-1/88
ID-3/88
TW/96P
TW/84C
BE-14/93
IT-121/92
NL-1/93
ES-1/93
DE-82/
VIIa
Pi_760/83
Pi_1168/84
Israel 70
Lebanon 70
Kuwait 256
Iraq AG68
AT-24/96
DK-1/95
CH-1/95
VI
NY parrot/70
CA 1085/71
HU-10/72
V
SG-4H/65
AF-2240
ZA-5/68
ZA-10/74
ZA-34/94
ZA-18/90
ZA-17/90
ZA-16/90
VIII
Herts 33/3
Italien/45
IV
Miyadera/51
AUS Victoria/32
III
Ulster 2C/67
V4 (Queensland)/66
I
LaSota (ZA)
LaSota/46
TexasGB/48
Beaudett C/45
II

Figure 5.11 Phylogenetic analysis of nucleotide sequence from the amplified 378nt RT-PCR fragment of the fusion protein gene. Phylogenetic relationships between isolates were determined at the Veterinary Medical Research Institute, Budapest using the program TREECON. The field strains and the LaSota vaccine strain used in this study are indicated in red.

Table 5.2 Nucleotide sequences for the deduced amino acid sequence at the F2/F1 cleavage site of strains positively identified as NDV and grouped into genotype VIIb.

Strain	Nucleotide sequence	Amino acid sequence	Virulence
M89/98 (Ostr. Pool 1)	AGG AGA CAG AAA CGC TTC	**R R Q K R F**	**V**
M57/98 (Av Pool AS)	AGG AGA CAG AAA CGC TTC	**R R Q K R F**	**V**
M308/98 (Av Pool)	AGG AGA CAG AAA CGC TTC	**R R Q K R F**	**V**
M89/98 (Av Pool 2)	GGG AGA CAG GGG CGC CTT	**G R Q G R L**	**L**

The above results show that strains M89/98 (Ost Pool 1), M57/98, and M308/98 are velogenic. They display the characteristic dibasic amino acid residues at the fusion cleavage site (Collins *et al.*, 1993). The presence of the phenylalanine residue after the point of cleavage is characteristic of velogenic, and mesogenic strains. This residue is replaced by a leucine residue in lentogenic strains (Collins *et al.*, 1993). The sequence observed at the fusion protein cleavage site of strain M89/98 (Av. Pool 2) is that characteristically displayed in lentogenic strains (Table 5.2). This strain also displayed embryo mortality indicative of lentogenic strains (Chapter 3).

It can be concluded upon comparison of the restriction enzyme profiles of the F protein amplimers of the field strains that they were not re-isolated forms of the vaccine strain LaSota. Strains 5.1 (M89/98) and 29.1 (M57/98) displayed restriction patterns described for genotype VIIb viruses. Strain 6.1 (M89/98, Av Pool 2), displayed a restriction fragment profile characteristic of group VIIb. However, cleavage products produced with *BstO O1* and *Hinf* 1 resulted in additional bands not observed in this group. This is thought to have arisen due to non-group specific cleavage. Epidemiologically, this could imply that mutations at various points within the sequence of the amplicon had taken

place. It should, however, be considered that not all the fragments released during cleavage are group specific. Varying banding patterns were produced when the F amplimer of strain 12.1(M308/98) was digested with *Hinf* 1. Digestion of the amplification product of this strain with *Rsa* 1 and *BstO O1* also produced large fragments not ascribed to this group. The overall restriction fragment profile suggests that it can preliminarily be grouped as a genotype VIIb virus.

Restriction fragment profiles obtained from digesting the matrix gene amplimers of the field strains were different from that observed for the positive control vaccine strain suggesting that these were not re-isolated vaccine isolates. Different banding patterns were observed among the group of field strains.

RT-PCR amplicons representing a region comprising the fusion protein cleavage site of field strains were successfully sequenced (Figures 5.7, 5.8, 5.9 and 5.10).

The dendogram (Figure 5.11) obtained from the analysis of the field strains under investigation grouped these isolates in genotype VIIb. These strains form a cluster closely related to isolates previously screened in 1993/1995 (Herczeg *et al.* 1999). The vaccine strain, LaSota, used as a positive control strain was grouped in genotype II, is closely related to LaSota/46.

Genotype VIIb strains are thought to have been introduced into South Africa and Mozambique by a single founder strain from the Far East (Herczeg *et al.*, 1999). This genotype is reported to be the most prevalent in South Africa, and has also been isolated in Southern Europe (Herczeg *et al.*,1999). Isolates grouped into genotype VIIb were isolated from South Africa in 1993, 1994 and 1995. These isolates were implicated in outbreaks in South Africa that started in the Gauteng Province. In this study strain M308/98 isolated from Sinoville, (Pretoria) in the Gauteng Province originated from the same region from which genotype VIIb isolates originated. Isolates M89/98 (Ostr Pool 1), and M89/98 (Av. Pool 2) where isolated from Rustenberg in the Northwest Province, neighbouring the Gauteng Province. After initial outbreaks in the Gauteng Province in

the early 1990s, genotype VIIb isolates spread to virtually the entire country, this is confirmed by the isolation of strain M57/98 from Graff-Reniet in the Eastern Province. Even though a small number of isolates were positively identified as NDV in this study, the fact that some of these isolates were collected from distant provinces, also confirms the observation that genotype VIIb isolates are the most prevalent in the region.

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CHAPTER 6:

GENERAL DISCUSSION AND CONCLUSIONS

Twenty-two viral strains purported to be NDV were collected from various veterinary institutions around South Africa. Thirteen strains were obtained from the Onderstepoort Veterinary Institute (OP), seven from the University of Pretoria (UP) Poultry Reference Laboratory, and two from EarlyBird Farms. Of the total number of strains investigated, only four of the field strains were positively identified as NDV using RT-PCR and/or HA/HI tests.

All the strains obtained from OP were allantoic fluid samples that arrived when they had already thawed. One could thus attribute the lack of infectivity to repeated freeze-thawing cycles. However, four of these strains survived these conditions, and were identified accordingly. Viruses vary in their susceptibility to heat, and most are heat labile (Burleson *et al.*, 1992). NDV however, is reported to be relatively heat stable, remaining infective for up to one month at room temperature (Burleson *et al.*, 1992). The medium in which viruses are contained should however be considered, as it has been reported that viruses are more stable during rehydration when they are in balanced salt solutions (Burleson *et al.*, 1992). During the few days of embryo development, allantoic fluid is essentially a physiological salt solution (Villegas, 1989). NDV is propagated in chick embryos 9-10 days old. At this stage the composition of allantoic fluid changes to pH 6.0 due to the presence of kidney excrements, and becomes turbid due to the presence of ureates (Villegas, 1989). The presence of ureates in the allantoic fluid often observable as white deposits, could have played a role in the destruction of viral protective structures, leading to the loss in infectivity. In such cases however, viral presence should still have been detectable by haemagglutination, as intact virions are not required for the observance of this phenomenon. This was not the case in the majority of the samples examined. The same argument applies to strains obtained from EarlyBird Farms, also contained in allantoic fluid. Of the two strains obtained from this company,

neither were found to be NDV. Negative results were obtained with both HA/HI tests and RT-PCR.

Field strains obtained from UP where lyophilized, and their lack of infectivity to a lesser extent could not be attributed to damage incurred because of storage. This storage mechanism is the most stable means of storing biological samples for long-term preservation. These strains did not agglutinate chicken erythrocytes. Indicating that the strains obtained were not NDV as indicated by the supplying laboratory.

Four strains from OP were successfully amplified upon RT-PCR of the fusion and matrix protein gene sequences of interest. An RT-PCR based detection technique was used by Herczeg *et al.*, in 1999 to group NDV isolates collected from South Africa and Mozambique during epizootics in the region in 1990-1995. These isolates were grouped into two novel groups VIIb and VIII. The grouping of these strains into these genotypes was based on RE analysis of a 75% region of the fusion protein gene, using restriction enzymes *Hinf I*, *Rsa I*, and *BstO* OI. The epidemiological survey used in this study was based on the application of this technique to screening NDV isolates collected from South Africa in 1998. Although RT-PCR is a relatively sensitive technique, the reaction components used for amplification had to be optimised as has been reported when RT-PCR protocols are transferred between different labs. RT-PCR Amplicons of field strains M89/98 (Ost Pool 1), M89/98 D (Av Pool 2), M308/98 (Av Pool), and M57/98 (Av Pool 1 1AS) were subjected to restriction enzyme analysis using the enzymes mentioned previously. RE profiles obtained overall were similar to those reported for genotype VIIb strains. However, fragments 517, and 519bp in size were observed upon the digestion of the F protein amplicon of strain M308/98 with *Hinf I.* The fragments occur in lentogenic isolates grouped in genotype II. This strain failed to kill chick embryos during passage indicating that it might be lentogenic. However, the amino acid sequence deduced at its fusion protein cleavage site was found to be typical of mesogenic-velogenic strains (RRQK/R↓F).

In addition, numerous fragments were observed in strains M308/98 and M89/98 (Av Pool 2) upon F amplicon restriction enzyme analysis using BstO I. These fragments are thought to be non-group specific cleavage products as the majority of the bands observed were characteristic of group VIIb isolates. Despite the appearance of non-group specific bands, with these strains their grouping into genotype VIIb along with strains M89/98 (Av Pool 1), and M57/98 was confirmed upon phylogenetic analysis.

RE analysis with *Hinf I* and MbO I of an amplicon obtained from amplifying an 88% region of the matrix protein gene can be used to differentiate two widely used vaccines LaSota, and B-1 (Wehmann, 1997). The matrix protein gene amplicons of the field strains were subjected to digestion with the two enzymes mentioned above. Common bands were observed between different isolates, however the overall banding patterns obtained were unique for each strain. Strain M89/98 (Av Pool 2) with a deduced amino acid sequence at the cleavage site typical of lentogenic strains (GRQGR↓L) also showed a banding pattern not reported for vaccine strains LaSota and B-1. This indicates that it is not a re-isolated form of the vaccine strains. This is further confirmed by phylogenetic analysis where this strain was grouped with genotype VIIb isolates. This strain is the only one of the field strains to contain a fusion protein cleavage site seen in lentogenic viruses. The other strains, M89/98 (Ost Pool 1), M57/98, and M308/98 had deduced amino acid sequences characteristic of velogenic strains (RRQK/RR↓F). This finding suggests that genotype VIIb isolates are heterogeneous in nature. Comprised of strains that vary in their degree of pathogenicity.

Genotype VII is comprised of NDV strains from different geographical areas, namely South Africa and the Middle East. South African isolates formed a distinct cluster termed VIIb, comprised of isolates collected during epizootics in 1993 and 1995 (Herczeg *et al.*, 1999). A genetic distance of 11% occurs between these isolates and those isolated from South Africa in 1990 and 1994 (Herczeg *et al.*, 1999). Phylogenetic analysis of the four field strains investigated, based on a variable portion of the F gene between nt 47-420 was performed, in order to determine the genotype to which the 1998 strains belong.

These strains grouped into genotype VIIb, along with strains previously isolated from South Africa (Fig 5.11). A 1.5%-1% divergence from the 1993, 1995 isolates was observed, which is consistent with a 0.5-1% divergence per year. It is thought that the VIIb epizootic in South Africa was initiated by a single founder strain, not indigenous to South Africa (Herczeg *et al.*, 1999). It is thought that the founder strain of cluster VIIb originated from the Far East (Herczeg *et al.*, 1999). These findings contradict the belief that the majority of recent ND outbreaks in South Africa are caused by endemic infections (Verwoerd, 1997). Recent outbreaks of ND in the region were caused by isolates of dual aetiology, one endemic to South Africa was grouped in VIII, and another that grouped in VIIb (Herczeg *et al.*, 1999).

Isolates belonging to genotype VIII were isolated earlier in the past decade (1990, and 1994), whereas those of VIIb seem to have been involved in more recent outbreaks (1993, 1995, 1998) (Fig 5.11). These results confirm the notion that a change in dominance of epizootic strains is taking place in South Africa (Herczeg *et al.*, 1999). It should however be considered that these observations could be as a result of unbalanced sampling in time and location as traditional epidemiological data is not available for this region.

The findings from this study are alarming, as highlighted in one particular case where an unknown pathogen (M193/98 Ost Pool 6) caused embryo death in 40 hours. The majority of the strains obtained from OP caused embryo death in a time span indicative of the presence of pathogenic agent. In addition strains M193/98 (Ost Pool 6), M183/98 (Ost Liv B), and M193/98 (Ost Pool 5) caused the agglutination of chicken erythrocytes. In all three cases, agglutination was not inhibited by anti-NDV serum. The results of the HA/HI tests, and in addition the lack of amplification with primers specific for NDV, led to a suspicion, of the presence of a different viral pathogen. This pathogen is thought to be avian influenza, as all three samples were collected from ostrich hosts, from which this virus has been previously isolated in South Africa (Bragg, personal communication).

The majority of the strains obtained from UP, and some from OP displayed embryo mortality characteristic of mesogenic NDV strains (Table 3.2). However, they failed to agglutinate chicken erythrocytes, and were not detected by RT-PCR, indicating that they are not NDV. These isolates are pathogenic as indicated by embryo mortality. The identification and characterisation of this/these unknown pathogen/s is beyond the scope of this project. It would, however, be interesting to determine their identity, as this would provide an informative report on the occurrence of the types of poultry pathogens present in South Africa.

In contrast this study also suggests that repeated outbreaks of disease in flocks thought to be protected against NDV by vaccination may in fact not be caused by NDV. The diagnosis of ND cannot be based on symptomatic observations alone. Routine laboratory tests, such as HA/HI tests should be included as part of diagnosing the occurrence of a certain aetiological agent during an outbreak.

It would be of great value if more virus samples of NDV are submitted for classification. This would serve the purpose of identifying populations of avian species harbouring these velogenic strains.

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SUMMARY

The primary objective in this study was the investigation of the molecular epidemiology of NDV strains isolated during epizootics in South Africa in 1998. Isolates purported to be NDV were collected from the Onderstepoort Veterinary Institute, the University of Pretoria, and EarlyBird Farms, Standerton. Four of the twenty-two isolates collected were identified as NDV, and successfully grouped into genotype VIIb, previously described by Herczeg *et al.*, (1999).

The isolates were propagated in 9-10 day-old embryonated SPF eggs. Embryo mortality was observed, allantoic fluid harvested, and genomic RNA extracted using TRIZOL® reagent, containing guanidine thiocyanate.

An RT-PCR based detection method was used to screen the isolates received (Ballagi-Pordány *et al.*, 1996). The optimised method entailed initial reverse transcription in a reaction mixture containing 1μM random hexamers $p(dN)_6$, 200 U Moloney Murine Leukemia (M-MLV) RT and 25 U RNasin. Aliquots of 5 μL of the cDNA mix were used as template in subsequent PCR amplification reactions.

A 1349bp region of the fusion protein gene was amplified using primers ONDV1aa and ONDV4aa. Fusion protein amplicons were obtained from field isolates, M89/98 (Ost Pool 1), M89/98 (Av Pool 2), M57/98, and M308/98 obtained from OP. Restriction enzyme profiles of the fusion protein amplicons using restriction enzymes *Hinf* I, *Bst*O I, and *Rsa* I displayed fragment patterns that allowed their grouping into genotype VIIb, described by Herczeg *et al.*, (1999). Non-group specific bands observed upon digestion of the amplicon of strain M308/98 with *Hinf* I released fragments not consistent with those observed in genotype VIIb isolates. These fragments suggest the presence of mutations in this area of the genome.

Amplification of a region comprising 88% of the matrix protein gene was facilitated using primers M1 and M2. These amplicons were subjected to RE analysis using

restriction enzymes *MbO* I and *Hinf* I. Analysis of the restriction profiles produced revealed that these four strains were not re-isolated forms of the commonly used live vaccine LaSota strain.

A region of the fusion protein gene encoding the fusion protein cleavage activation site was amplified by means of a nested PCR, and sequenced. Initial PCR amplified a region spanning from the M gene nt 778 to F gene nt 545 using primers K1 and K2. These amplicons were purified and used as template in a nested PCR using primers MV1 (M gene nt 1163) and B2 (F gene nt 470).

Sequence analysis revealed that the amino acid sequence at the fusion protein cleavage site of strains M89/98 (Ost Pool 1), M57/98,and M308/98 was RRQKR ↓ F, indicative of velogenic strains. Strain M89/98 (Av Pool 2) displayed a sequence at the fusion protein cleavage site that is characteristic of lentogenic strains GRQGR↓L. This finding suggests that genotype VIIb isolates are heterogeneous, composed of strains of varying pathogenicity.

Phylogenetic analysis based on a 378nt long region of the nested PCR amplicon allowed the grouping of all these isolates into genotype VIIb. A 1.5-1% divergence was observed between group VIIb isolates collected in 1993, and 1995, and those used in this study, collected in 1998. This genetic distance is consistent with a 0.5-1% divergence in strains per year.

The remaining field strains not detected by RT-PCR, but that displayed embryo mortality indicative of pathogenic agents were subjected to HA/HI tests. Strains M193/98 (Ost Pool 6), M183/98 (Ost Liv B), and M193/98 (Ost Pool 5) agglutinated chicken erythrocytes. Haemagglutination was not inhibited by anti-NDV serum. These results led to the suspicion of the presence of an unidentified pathogen, most likely avian influenza, as all three samples were collected from ostrich hosts, from which this virus has previously been isolated in South Africa.

OPSOMMING

Die primêre doelwit van hierdie studie was om stamme van die epidemologiese Newcastle-siekte virus (NDV) wat tydens gelokaliseerde uitbreke van Newcastle-siekte in Suid-Afrika gedurende 1998 geïsoleer is, op molekulêre vlak te ondersoek. Sogenaamde NDV isolate is vanaf Onderstepoort Veeartsenykunde Instituut, Universiteit van Pretoria en Early Bird Plase, Standerton verkry. Slegs vier van die twee-en-twintig isolate kon as NDV geïïndentifiseer word en suksesvol as genotipe VIIb volgens beskrywing deur Herczeg *et al.* (1999) geklassifiseer word.

Vermeerdering van die isolate is bewerkstellig deur kweking in 9-10 dag-oue bevrugte spesifiek patogeenvrye (SPV) eiers. Die tydperk voor die afsterwing van die embrio is aangeteken. Hierna is allantoïse vloesftof uit die eier onttrek en totale RNA met behulp van guanidien tiosianiedbevattende TRIZOL® geïsoleer. Sifting van die isolate is bewerkstellig deur middel van die tru-transkriptase polimerase kettingreaksie (RT-PCR) metode (Ballagi-Pordány *et al.*, 1996).

In die geoptimiseerde RT-PCR metode is die boodskapper RNA (mRNA) *in vitro* omgeskakel na cDNA in 'n reaksiemengsel wat 1 µM ewekunsige heksanukleotiede, $p(dN)_6$, 200 eenhede Moloney Murien Lukemia Virus tru-transkriptase (M-MLV RT) en 25 eenhede ribonuklease stremmer (RNasin) bevat het. 5 µl hoeveelhede van die gevormde cDNA is as substraat vir die daaropvolgende polimerase kettingreaksies (PKR) gebruik.

Inleiers ONDV1aa and ONDV4aa is gebruik om 1349 basispare (bp) van die fusieproteïengeen van die virus te amplifiseer. Fusieproteïen PKR-produkte is verkry vir veld-isolate M89/98 (Ost Pot I), M89/98 (Av Pot 2), M57/98 en M308/98, verkry vanaf Onderstepoort. Ontleding van *Hinf* I, *BstO* I, en *Rsa* I restriksie-ensiemsnydings van hierdie fusieproteïen PKR-produkte het daartoe aanleiding gegee dat die isolate waarvan dit verkry is as genotipe VIIb geklassifiseer kon word soos beskryf deur Herczeg *et al.* (1999). Die nie-groep VIIb spesifieke fragmente wat waargeneem is nadat die PKR

produk van stam M308/98 met *Hinf* I verteer is, het tot die gevolgtrekking gelei dat moontlike mutasies in hierdie gebied van die genoom van M308/98 voorkom.

Amplifikasie van 'n gebied wat 88% bestaan, is deur middel van PKR met behulp van inleiers M1 en M2 verkry. Ontleding van die fragmente verkry na *MbO* I en *Hinf* I restriksie-ensiemsnydings van die onderskeie PKR-produkte, het daarop gedui dat die betrokke stamme nie hergeïsoleerde vorms is van die LaSota stam wat in lewende entstof gebruik word nie.

Deur middel van beskutte inleierbenadering is die gebied van die fusieproteïngeen wat vir die fusieproteïen-splytingsaktiveringsetel kodeer, geamplifiseer en die basisvolgorde daarvan bepaal. Die eerste stel inleiers, K1 en K2, het amplifisering vanaf nukleotied 778 van die M-geen tot nukleotied 545 van die F-geen ingelei. Hierdie PKR-produkte is gesuiwer en as substraat gebruik met die tweede stel inleiers MV1 en B2 wat tot amplifisering vanaf nukleotied 1163 van die M-geen tot nukleotied 470 van die F-geen tot gevolg gehad het.

Die aminosuurvolgorde soos afgelei uit die nukleotiedvolgorde van die fusieproteïen-splytingsaktiveringsetels van stamme M89/98 (Ost Pot I), M57/98 en M308/98 was RRQKR ↓ F wat aandui dat hierdie stamme velogenies is. Die aminosuurvolgende, GRQGR ↓ L, van die fusieproteïen-splytingsaktiveringsetel van stam M89/98 (Av Pot 2) was kenmerkend van lentogeniese stamme. Hierdie bevinding dui daarop dat genotipe VIIb isolate heterogeen is en dat die groep saamgestel is uit stamme met wissselende patogenisiteit.

Filogenetiese ontleding, gegrond op die 378 bp fragment verkry met beskutte inleierbenadering, groepeer die genoemde isolate in genotipe VIIb. Die groep VIIb isolate wat in 1993 en 1995 versamel is, toon 'n afwyking van 1-1.5% ten opsigte van die isolate wat in 1998 versamel en in hierdie studie gebruik is. Hierdie genetiese afstand is in ooreenstemming met 'n 0.5-1% afwyking in stamme per jaar.

Die oorblywende veldisolate wat die dood van embrio's net soos die patogeniese agente kon veroorsaak, maar wat nie deur middel van tru-transkriptase PKR opgespoor kon word nie, is aan HA/HI toetse onderwerp. Stamme M193/98 (Ost Pot 6), M183/98 (Ost Liv B) en M193/98 (Ost Pot 5) het 'n agglutinasie van hoender rooi-bloedselle veroorsaak. Haemagglutinasie was nie deur die byvoeging van anti-NDV serum vertoed nie. Hierdie resultate het die vermoede laat ontstaan dat 'n ongeïdentifiseerde patogeen teenwoordig was, waarskynlik voëlgriepvirus, veral omdat al drie hierdie monsters vanaf volstruisgashere versamel is, waaruit hierdie virus al voorheen in Suid-Afrika geïsoleer is.