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Molecular epidemiology of Newcastle disease virus In Australia

An analysis of the genome of Australian Newcastle disease viruses to better understand the epidemiology of virulent disease outbreaks and their control

A report for the Rural Industries Research and Development Corporation

by Dr. Allan R. Gould

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Researcher Contact Details
Dr. Allan R Gould
CSIRO, Livestock Industries
Australian Animal Health Laboratory
PO Bag 24
Geelong, 3220 Australia

Phone: 03 52275119
Fax: 03 52275555
Email: allan.gould@csiro.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4819
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au.
Website: http://www.rirdc.gov.au

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Foreword

With the emergence of virulent Newcastle disease at Dean Park and Mangrove Mountain (New South Wales) in 1998, it was vitally important to discover where this virus had come from, its disease potential and the likelihood of it continuing to cause outbreaks of virulent NDV in industry. It was a priority to quickly establish molecular based techniques to rapidly diagnose the presence of a Newcastle disease virus in affected birds and to determine if the genomic sequences carried by that virus were virulent, to aid in the eradication of diseased animals. Therefore, the aims of this project were to attempt a classification of the types of Newcastle disease virus (NDV) present in Australia and to gain an understanding of the interactions or prevalence of these viruses within the poultry industry. It was also deemed important to delineate the regions of the NDV genome responsible for the emergence of virulent disease in the field, and to investigate the parameters behind the emergence of virulent NDV in the poultry industry.

This report details the use of a DNA amplification-DNA sequencing methodology that enabled a viral genetic sequence to be determined from infected tissue or allantoic fluid in 18 hours or less. This rapid analytical technique at the genome level of the virus was crucial to effective decision making for control/quarantine of the virus in the field.

The project also continually monitored the strains of virus present in Australia during subsequent outbreaks of virulent virus in 1998, 1999 and 2002. Research undertaken also involved the genetic analysis of the 'progenitor' strain of NDV from which the virulent virus emerged, and followed its gradual genetic evolution from 1998 to 2002.

Phylogenetic and quasi-species analysis of virulent and avirulent NDV field isolates, enabled mechanisms to be proposed whereby an avirulent virus can infect a bird and subsequently release virulent virus. These experiments may form an important basis for the understanding of the mechanisms whereby virulent NDV outbreaks occur in the field.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, a new addition to RIRDC's diverse range of over 1,000 research publications, forms part of our Chicken Meat R&D program, which aims to support increased sustainability and profitability in the chicken meat industry by focusing research and development on those areas that will enable the industry to become more efficient and globally competitive and that will assist in the development of good industry and product images. Funding was also provided by the RIRDC Egg Program, now the Australian Egg Corporation Limited.

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Simon Hearn
Managing Director
Rural Industries Research and Development Corporation

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In addition to the funds received from RIRDC and AECL, CSIRO Livestock Industries, Australian Animal Health Laboratory, also provided financial, personnel and laboratory support for the project.

Abbreviations

CEF	chick embryo fibroblast
F	fusion gene
HN	haemagglutinin-neuraminidase
IBDV	infectious bursal disease virus
IBV	infectious bronchitis virus
ICPI	intracerebral pathogenicity index
L gene	large gene (RNA dependant RNA polymerase)
M	matrix
N	nucleocapsid
ND	Newcastle disease
NDV	Newcastle disease virus
P	phosphoprotein
PCR	polymerase chain reaction
RT-PCR	real-time PCR
SPF	specific pathogen free

Glossary

Ancestor virus	The ND virus which gave rise to the progenitor virus.
Clade	A group of viruses that are related in their nucleotide sequence as revealed by a phylogenetic analysis.
Consensus sequence	In terms of quasi-species concepts (see below) applying to RNA viruses, the consensus sequence provides a representation of the populations' sequence (at the nucleotide level), not that of the individual viral genomes making up the population, since each virus genome sequence can and probably will be different at some location(s) from the consensus sequence.
Polymerase chain reaction	A reaction in which a small portion of RNA or DNA is amplified using synthetic oligonucleotides and a heat resistant DNA polymerase to produce multiple copies of DNA. These DNA copies are then used to obtain the DNA sequence of the amplified segment.
Progenitor virus	The virus from which the virulent Australian NDV emerged. The type virus of these progenitor viruses is 98-1154 (isolate MM-98-1).
Quasi-species	In the quasi-species model of RNA virus genomes, mutations occur through errors made in the process of copying already existing RNA genome sequences. Due to the ongoing production of mutant sequences during the replication of the RNA virus genome, the virus population is made up of a "mutational cloud" of closely related sequences, referred to as quasi-species.
Real time PCR	A quantitative method of estimating the presence of DNA amplicons in a PCR reaction using fluorescent probes.
Virulent	In the context used throughout this report, **virulent** describes an NDV with a fusion cleavage site of RRQRRF (where R=a basic amino acid), while an **avirulent** virus is defined as having one or other alterations in the dibasic amino acid motifs (generally to a G residue) and/or the lack of the F amino acid i.e. RRQRGL.

Contents

Executive Summary

Major objectives of the project

The research undertaken had the following primary objectives:

- To develop a better understanding of the epidemiology of NDV in Australia;
- To determine the cause of the virulent Newcastle disease virus outbreaks in Dean Park (NSW, 1998), Mangrove Mountain (NSW, 1998-2000) and Meredith (Victoria, 2002) and to characterise the viruses responsible for these outbreaks.
- To develop rapid molecular diagnostic procedures to detect and characterise the genetic sequence of NDV isolates.
- To develop a better understanding of the mutation rates as well as disease potential of Australian NDV isolates and determine some of the factors influencing the genome of Australian NDVs which lead to the emergence of virulent virus and thus potentially to an outbreak of NDV in the field.

Background and methods

The project was initiated in response to the outbreak of virulent Newcastle disease virus that initially appeared at Dean Park in NSW in 1998. At that time, it was not known whether the outbreak was caused by the incursion of an exotic overseas NDV into Australia or by a virus already present in Australia.

Subsequent to the initial outbreak, virulent virus was also detected at Mangrove Mountain in NSW, also in 1998. This outbreak continued until 2000, even in the presence of a program involving quarantine and slaughter of affected poultry. During the period of this project, virulent NDV and the progenitor virus was also detected in Orchard Hills, Glenorie, Marsden Park, Tamworth, Appin and Rossmore. A separate outbreak occurred at Meredith (Victoria) in 2002 and most recently in Horsley Park, NSW (2002).

The research undertaken aimed to fully characterise the viruses responsible for virulent NDV in the poultry industry in Australia and to develop new and improved diagnostic procedures involving amplification of the genome of the virus responsible for the disease. A procedure was finally adopted whereby it was possible to proceed from infected poultry tissue to the detection and determination of the virulent viral sequences within 12-18 hours using the polymerase chain reaction (PCR) and rapid sequencing methodologies. The virulence of the ND virus was assessed using amino acid sequence parameters of the fusion cleavage signal in the fusion protein. Gene sequence was also determined from other regions of the NDV genome to attempt phylogenetic characterisation of the viruses so that the lineage or ancestry of these viruses could be determined. One such location was obtained which enabled the assignment of NDV to different families within those known to circulate in Australia.

It should be noted that in the context used throughout this report, **virulent** describes an NDV with a fusion cleavage site of RRQRRF (where R=a basic amino acid); while an **avirulent** virus is defined as having one or other alterations in the dibasic amino acid motifs (generally to a G residue) and/or the lack of the F amino acid i.e. RRQRGL.

Molecular characterisation of Australian NDV isolates

To obtain a better understanding of the viruses responsible for the outbreaks of virulent NDV in Australia and to set a bench mark for further research studies, the complete genome of the virulent and avirulent ‘progenitor’ viruses involved in the Dean Park, Peats Ridge (Mangrove Mountain area) and Meredith outbreaks were completely sequenced. Some variant viruses that occurred during the outbreaks of NDV in these areas were also completely sequenced and the biological properties of these isolates investigated in poultry. In all, ten complete genome sequences were determined and differences noted in the rate of mutation for certain genes.

Molecular analyses based on the study of the genomic sequence(s) of circulating Australian and overseas NDVs showed that the virus that was the cause of the initial outbreak of Newcastle disease was related to a previously described avirulent NDV isolated from Peats Ridge in the Mangrove Mountain area in 1998, rather than the incursion of an exotic overseas virulent virus. The evolution of the virus responsible for the outbreak of virulent NDV in Australia (termed the 'progenitor' virus) was also characterised at the molecular level. From these studies it was shown that the 'progenitor' virus was slowly evolving, as determined from its nucleotide sequence at its initial isolation in 1998 to that seen in 2001. However, analyses of these genome sequences demonstrated that the 'progenitor' virus did not appear to have a fusion cleavage signal sequence, which determines the virulence of the virus, and was prone to greater genetic variation than any other region in its genome.

Comparison of the 'progenitor' virus with overseas and earlier Australian isolates

The 'progenitor virus' had some unique characteristics which were to become important in subsequent investigations. The nucleotide sequence of its fusion (F) gene was initially observed to be only two nucleotides different from that of a virulent virus, and the haemaglutinin-neuraminidase (HN) gene had an extension of nine amino acids when compared to the normal amino acid sequence for this protein. Comparison with Australian isolates and those from overseas showed it to be related to Australian NDV isolates and not overseas isolates. In particular, two isolates which were isolated in NSW in 1986 and in Queensland in 1987 were shown to have a genetic relationship with both the 'progenitor' virus and with the virulent isolates made in NSW and Victoria (1998-2002). From a retrospective survey of NDV isolates, this 'ancestor' virus to the 'progenitor' and virulent viruses was demonstrated to have been present at Mangrove Mountain NSW in 1991.

Analysis of individual virus sequences comprising field isolates (quasi-species analysis)

Gene sequence analysis of the individual viruses present in a field isolate of NDV was done to investigate the genetic variability present in these isolates. It was determined that, for a defined region of 500 nucleotides over the fusion gene cleavage site, approximately 50% of the individual viruses that made up the field isolate had the same nucleotide sequence as that determined to be the sequence of the field isolate. However, the remaining individual viruses contained mutations. These mutations occurred randomly in either the first, second or third nucleotide of a codon for an amino acid. The latter usually did not result in the alteration of the amino acid sequence of the protein, however the other two mutation positions generally resulted in the generation of an amino acid change. These amino acid changes were predominantly conservative substitutions; however some radical changes were observed.

Gene sequence analysis showed that some field isolates of NDV with a relatively high intracerebral pathogenicity index (ICPI) had an avirulent F cleavage site. Quasi-species analysis of these field isolates showed that virulent virus was present up to a level of 26% of the viral population. However, poultry infected with these isolates behaved as if they were infected with an avirulent NDV. Real-time PCR demonstrated that, in some isolates of progenitor virus, virulent NDV sequences were present at a low level of 1:1,000 to 1:5,000.

Experiments which analysed the error rate as well as the total variants within a quasi-species showed that only two to three rounds of replication, *in vivo* or *in vitro*, are necessary for a plaque-purified virus to approximate the level of variation of the viral population seen in field isolates.

Detection of virulent virus in field isolates using tissue culture breakthrough

Plaque-purified avirulent NDV grown in tissue culture demonstrated the emergence of virulent virus by mutation after a period of six to seven days. The level of virulent virus that emerged after tissue culture was shown to vary from 1-18%. Virulent virus was also demonstrated to be present in field isolates of NDV isolated in Australia in the 1980s which belonged to a separate clade from those associated with the 1998-2002 NDV outbreaks. These latter viruses where shown to have HN extensions of seven amino acids. The presence of these virulent viruses in an avirulent background remains undetected until a selection pressure is applied.

Association of HN amino acid extension with respiratory disease in poultry

Sequence analysis of a number of overseas NDV isolates has shown that the HN protein is normally 571 amino acids in length; however, Australian NDV isolates have an additional 45 amino acids at the C-terminus of the HN protein. Sequence analyses of many Australian isolates as part of this study showed that the C-terminal extensions of the HN gene can vary from 7-45 amino acids. Australian NDV isolates with HN extensions of 45 amino acids generally replicate in the gut of the bird while those with HN extensions of 14, 9 and 7 amino acids have been associated with "late summer respiratory disease" syndrome and virus replication in the lungs and respiratory tract. The viruses generally associated with this syndrome at present in Australia have 9 or 14 amino acid extensions to their HN proteins.

Factors affecting the emergence of virulent virus from an avirulent inoculum in poultry

Experiments were undertaken *in vivo* to determine if the presence of a pre-existing avian pathogen could influence the emergence of virulent ND virus from an avirulent virus. Preliminary trials have shown that the presence of the coronavirus, infectious bronchitis virus (IBV), during an NDV infection does not alter the apparent symptoms nor virulence of NDV during infection. However the infection of birds with infectious bursal disease virus (IBDV) prior to NDV infection may exacerbate NDV disease symptoms, as well as potentially allowing virulent virus, which has arisen by spontaneous mutation(s), to dominate the quasi-species population replicating in poultry.

Conclusions and final recommendations

Australia has currently circulating a wide spectrum of NDV isolates that have now been well characterised at the molecular level. Some well-defined molecular properties have been ascribed to loci within the genome, the most important of these being the virulence sequence at the fusion protein cleavage site. Another is the possible link between the HN amino acid extension and viral replication in the respiratory tract.

Over the period of this project, virulent NDV continued to emerge from an avirulent NDV background despite the implementation of quarantine control measures. Although V4 has proved effective as a vaccine, the temporal and geographic utilisation of V4 may need to be co-ordinated with a screening program to detect and eliminate the presence of the 'progenitor' virus.

The emergence of virulent virus in Australia is well connected to the presence of an avirulent 'progenitor' virus that, through natural mutation processes, can harbour virulent virus within its quasi-species. This virulent virus will not emerge from the avirulent background unless a selection pressure is placed on the quasi-species.

Another clade of NDV has been described which is also two nucleotides from virulence and has the same potential to initiate virulent NDV outbreaks in Australia. This clade is characterised by having a 7 amino acid HN extension. Virulent virus has been isolated from these field isolates.

It is recommended that a detailed survey be conducted within Australia to detect the presence of the 'progenitor' virus and eradicate it before the virus is spread further and causes further outbreaks. The role of other viral and environmental factors in the emergence of virulent virus from the 'progenitor' virus also needs to be further evaluated.

1. Introduction

Newcastle disease virus (NDV) is classified as a member of the family Paramyxoviridae in the order Mononegavirales. Of the two subfamilies, the Pneumovirinae and the Paramyxovirinae, NDV is placed within the Rubulavirus genus of the latter subfamily. The genome comprises a single-stranded (ss) negative sense RNA of approximately 15.2 Kb that codes for an RNA directed RNA polymerase (L gene), haemagglutinin-neuraminidase (HN gene), fusion (F gene), matrix (M gene), phosphoprotein (P gene) and nucleocapsid (NC gene) proteins in that order, from the 5' terminus to the 3' terminus.

NDV strains can be classified as highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic) on the basis of their pathogenicity for chickens. The molecular basis for these differing levels of pathogenicity appears to be mainly determined by the amino acid sequence motif present at the protease cleavage site of the precursor fusion protein (F_0) and the ability of cellular proteases to cleave the F_0 protein of different pathotypes (Nagai *et al*., 1976a; Ogasawara *et al*., 1992). This has been clearly demonstrated by Peeters *et al.* (1999) in which a recombinant lentogenic NDV had the F_0 protein cleavage site altered from GRQGR↓L to RRGRR↓F, which resulted in a shift in pathogenicity from a completely non-virulent NDV to highly virulent NDV. Another characteristic of NDV isolates is the ability of the HN protein to have a variable amino acid extension at their carboxy-terminus (C-terminus) which can vary from 0 to 45 amino acids in the case of the Albiston-Gorrie or V4 isolates, respectively (Sakaguchi *et al*., 1989). HN proteins are type II integral membrane proteins spanning the membrane once, thus the amino-terminus (N-terminus) which contains a single hydrophobic domain acts as a combined signal/anchor domain leaving the C-terminus extracellular in virus-infected cells. The function of this C-terminal variability is unknown but it could be speculated that it is involved with virus-cell fusion in conjunction with the F protein (Lamb and Kolakofsky, 1996). However, for some strains of NDV, HN is synthesized as a biologically inactive precursor from which 90 amino acids are removed from the C-terminus to activate the molecule (Nagai *et al*., 1976b; Nagai and Klenk, 1977).

Australia was involved in the first panzootic of NDV in which exotic virulent virus was detected in chickens in Melbourne, Victoria, in 1930 (Johnstone, 1933) and 1932 (Albiston and Gorrie, 1942). The strain responsible for this outbreak was the Albiston-Gorrie strain which had an F_0 cleavage signal of RRQKR↓F and an HN C-terminal extension of 0 amino acids (KDDGV)(Collins *et al*., 1993; Hansson and Della-Porta, unpublished). The outbreak of disease was controlled by a combination of quarantine and slaughter and it was not until the mid 1960s that it was recognised that Australia had its own endemic, avirulent strains of NDV, characterised by the Queensland D26 strain or V4 (Simmons, 1967). More recently, these strains have been characterised at the molecular level and shown to have avirulent F_0 cleavage sites (GKQGR↓L) and HN extensions of 45 amino acids (Sato *et al*., 1987; Toyoda *et al*., 1989). A more recent development has been the reports of Hooper *et al*. (1999a) in which certain Australian strains of NDV were shown to be involved with a respiratory form of the disease. However these viruses were still considered to be of low virulence (Hooper *et al*., 1999b). In 1998 the first occurrence of virulent NDV since the 1930s occurred in New South Wales at Dean Park in 1998 (Gould et *al*., 2001).

2. Objectives

This research was undertaken to deliver the following outcomes:

- A better understanding of the epidemiology of NDV in Australia.
- The ability to predict possible progenitor viruses for virulent NDV outbreaks within Australia.
- A better understanding of the mutation rates as well as disease potential of Australian NDV isolates at the molecular level.

In order to deliver these outcomes, the specific objectives of the research undertaken were to:

- characterise at the molecular level the range of Australian NDV circulating in the poultry industry;
- determine molecular loci that could predict the origin of a particular isolate;
- develop rapid molecular diagnostic techniques that could identify and characterise an NDV isolate;
- determine the complete sequence of a number of NDV isolates associated with Australian NDV outbreaks for comparative studies and to generate a database for further studies on genetic drift and mutation rates;
- develop a real-time PCR diagnostic assay to detect virulent NDV;
- analyse the mutation rate of NDV isolates associated with disease outbreaks; and
- determine which factors can influence the emergence of virulent NDV in the field.

3. Methodology - General

3.1 Collection and origin of samples

NDV isolates were generally submitted to the Australian Animal Health Laboratory for disease diagnosis during the course of normal disease investigations within Australia. Other viral isolateswere obtained from the collections of Drs M. MacKenzie (Ingham's Enterprises) and G. Burgess (James Cook University). Virus isolation, tissue preparation and nucleic acid extraction were from 10% homogenates or formalin-fixed tissues as described by Hooper *et al*. (1999a and b).

Virus isolates used in the present study were as follows:

PR-9/98-1 and PR-9/98-2, isolates of NDV progenitor virus from Peats Ridge, Mangrove Mountain, made in September 1998 (AAHL Accession #'s, 98-1154#4 and 98-1154#6);

MM-3/99, MM-4/99 and MM10/99, NDV isolates from Mangrove Mountain made in March, April and October 1999, respectively (AAHL Accession #s 99-0530; 99-0655; 99-0963-1, respectively);

OH-1/00, Orchard Hills isolate from January 2000 (AAHL Accession # 00-0001);

DP-9/98-1 and DP-9/98-2, isolates from Dean Park September, 1998 (AAHL Accession # 98-1252 and 98-1235, respectively);

GN-9/98-1 and GN-9/98-2 isolates from Glenorie September, 1998 (AAHL Accession # 98-1238-1 and 98-1238-2, respectively);

MP-1/00, Marsden Park isolate, January 2000 (AAHL Accession #00-0127);

TAM-1/00 isolated Tamworth, January 2000 (AAHL Accession # 00-0046);

RM-2/001 and RM-2/00-2 isolates from Rossmore in February 2000 (AAHL Accession # 00-0347 and 00-0346, respectively);

AP-2/00 isolate from Appin, February 2000 (AAHL Accession # 00-0196);

MM99-1014, V4 like NDV isolated Mangrove Mountain October, 1999 with 14 amino acid extension sequence (AAHL Accession # 99-1014);

B1-smugg/97, B1 vaccine strain smuggled into Australia from Taiwan (1997, AAHL Accession # 97-0788);

Viet-1/97, Viet2/97 and Viet-3/97 are virulent Vietnamese NDV isolates (1997, AAHL Accession #s 97-1453 [6-8]).

Other virus isolates are:

AV32 (Albiston Gorrie isolate, 1930-32; GenBank Acc #M22110, M24712);

NSW12/86* (isolated Newcastle, NSW, 1986);

Qld 1/87 (isolated Queensland, 1987);

V4* (isolated Queensland, 1966; GenBank Acc #s AF217084 and J03911);

ITA-45 (GenBank Acc #M24715);

B1-A (GenBank Acc # U22266);

Las-46 (GenBank Acc #M24709);

HER33 (GenBank Acc #M24714);

Essex-70 (GenBank Acc #U52448);

CHI-85 (GenBank Acc #M24716);

90-0028 (isolated Red Hill, Victoria, 1990);

B1-47 (GenBank Acc # M24708);

B1-2 (GenBank Acc #U37188);

B1-3 (GenBank Acc #U37189);

In333 and In337 (Indonesian isolates, 1987);

D26-76 (GenBank Acc #M24705);

Ulster-67 (GenBank Acc #M24707);

ULSTER-2 (GenBank Acc #M19478);

King-2* (Victoria, 1991); Bryant-1 (Victoria, 1991);

V315 (NSW, 1978); Moura-1 (Queensland, 1992-Goose isolate);

Moura-2 (Queensland, 1992-Duck isolate);

Wa1886 (Western Australian black duck isolate, 1978).

Other NDV isolates are indicated by their GenBank accession numbers; those not assigned a GenBank accession number are described in Della-Porta *et al.* (2000, submitted). Viruses labelled * are described in Hooper *et al.* (1999a).

3.2 PCR amplification and sequence analysis

Primary PCR was done on extracted nucleic acid from either fresh or paraffin embedded or formalin fixed tissues as described by Hooper *et al.* (2000). For PCR amplification of F gene sequences, the primers and conditions developed by Collins *et al.* (1993) were used for both primary and hemi-nested PCR reactions. PCR fragments were amplified from HN gene sequences using RT-PCR and the following primers: NDV HN– 314 5'-ATATCCCGCAGTCGCATAAC; NDV HN-304 5'-TTTTTCTTAATCAAGTGACT and NDV HN-330 5'-GCAGCATACACAACATCAACATG.

Primary PCR was done with primers HN-304 and HN-330 using reaction conditions of 94^{o}C for one minute, annealing at 37^{o}C for two minutes and an extension time of two minutes at 72^{o}C for 35 cycles. Hemi-nested PCR reactions were done using the primers HN-330 and HN-304 and using the same reaction conditions except the number of cycles was reduced to 25.

DNA amplified from these reactions was purified after electrophoresis through 1% agarose gels using QIAquick DNA preparation columns (Quiagen Inc, USA) according to the manufacturer's instructions. Purified DNA was then sequenced using the BigDye Terminator cycle sequencing and ABI 377 automated DNA sequencer (Perkin Elmer Applied Biosystems).

Sequences were compared using ALIGN Plus and Clone Manager programs (Scientific and Educational Software) and phylogenetic analyses done using Clustal V (PCGene suite of programs, Intelligenetics) or the PHYLIP programs (Felsenstein, 1993) using Protpars and DNApars were used to estimate phylogenies using the parsimony method. Phylogenetic trees are displayed using the Treeview program (Page, 1996).

4. Studies Undertaken and Results

4.1 Gene sequence analysis of NDV

Fusion gene

Gene sequence analyses were done on a 150bp region covering the fusion cleavage site of Australian NDV isolates of viruses involved with the 1998-2000 outbreaks and these were compared to the sequences of other Australian and overseas NDV isolates (Figure 1). As expected, viruses from the Dean Park, Mangrove Mountain and Tamworth areas all clustered together with essentially the same sequence; this sequence being different from other Australian and overseas isolates. Phylogenetic analyses (Figure 2) of these sequences showed that the wild bird isolates were also markedly different from those isolated from poultry and formed a distinct and separate clade. However other regions of the genome were also investigated for their ability to reflect inter-relationships between viral isolates.

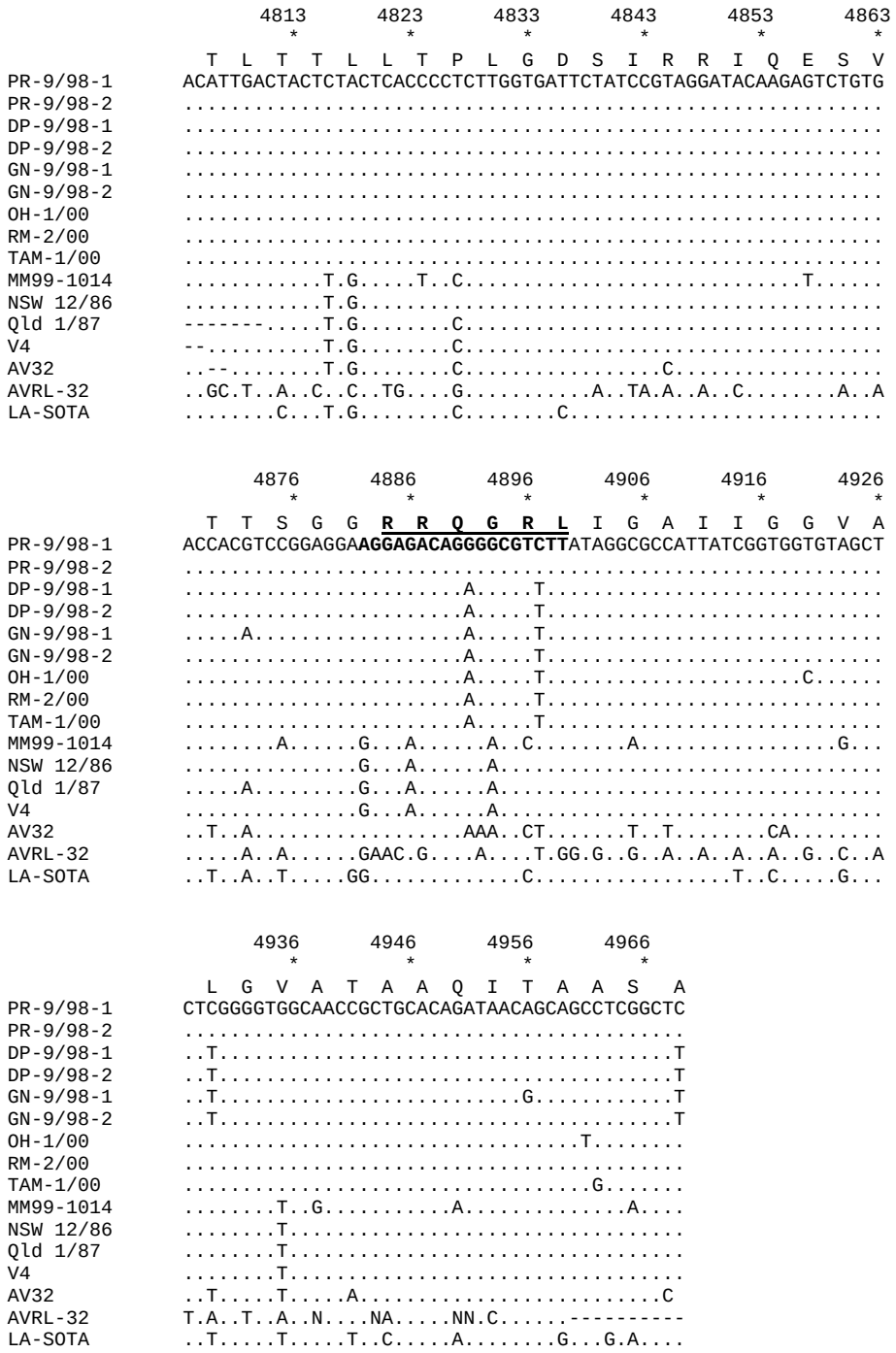

Figure 1. Fusion cleavage site gene sequence analyses of Australian and overseas NDVs

Notes: Numbering is relative to the complete genome sequence of NDV (Peeters *et al*., 1999; Römer-Oberdörfer *et al*., 1999). The coding sequence for the fusion cleavage sequence is emboldened. Non sequenced regions are indicated with an (-) while undetermined nucleotides are indicated by an N.

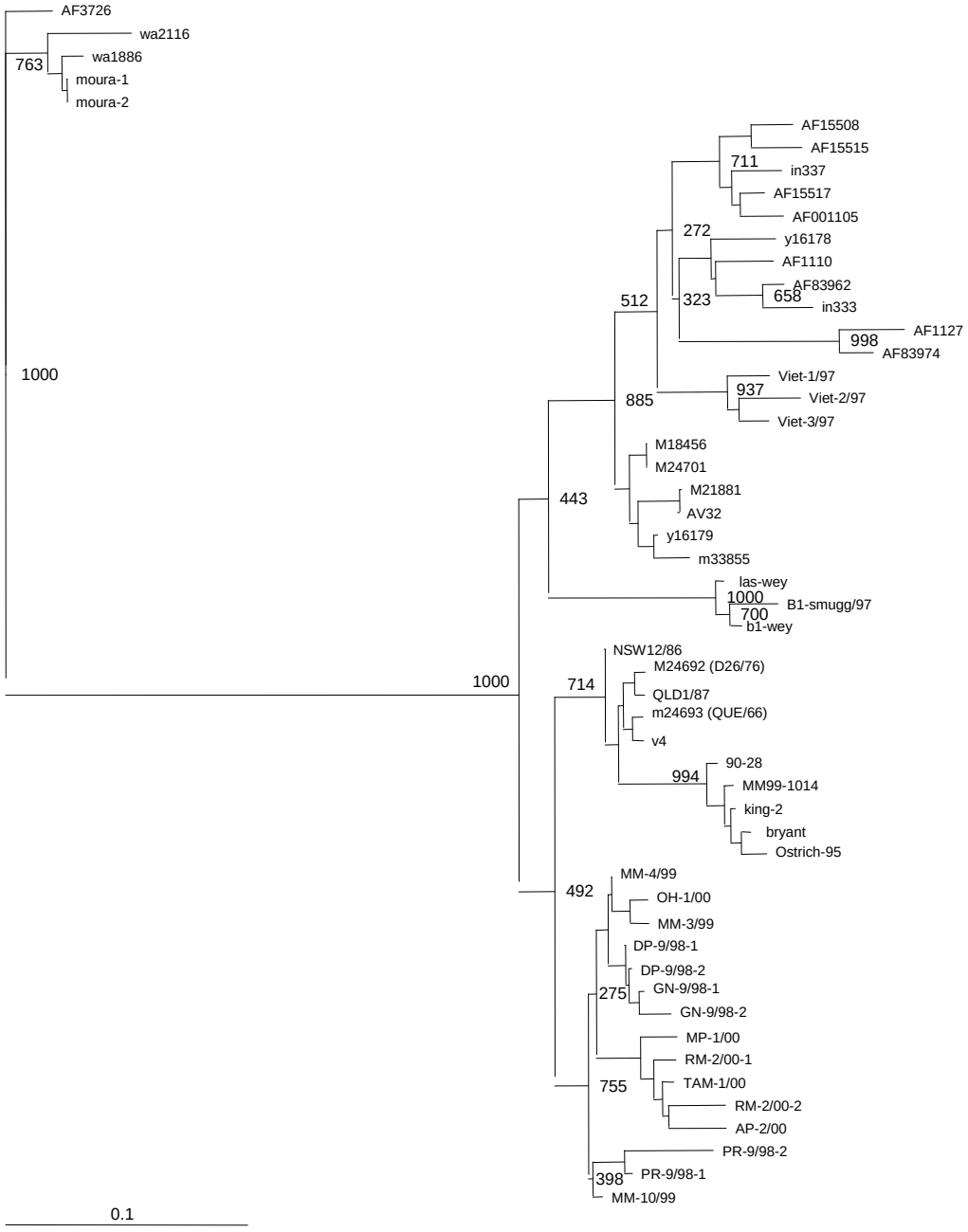

Figure 2. Phylogenetic analysis of fusion (F) gene cleavage sequences for Peats Ridge progenitor virus isolates compared to isolates from the 1998-2000 NDV outbreaks.

Notes: Isolates are as indicated in Materials and Methods. Sequences covering nucleotides 4732-4980 of the genomic sequence were compared for phylogenetic analyses and a bootstrap analysis of 1000 replicates is presented as a rooted tree with NDV wild bird isolates used as the outgroup.

Haemaglutinin-neuraminidase gene

Nucleotide sequences were obtained by sequence analysis of PCR amplified DNA covering the carboxyl terminus of the HN gene and the intragenic region between the HN and L gene products. Again, comparison of these sequences showed a clustering of the Australian outbreak viruses which were distinct from those of other Australian viruses and overseas NDV isolates (see Figure 3).

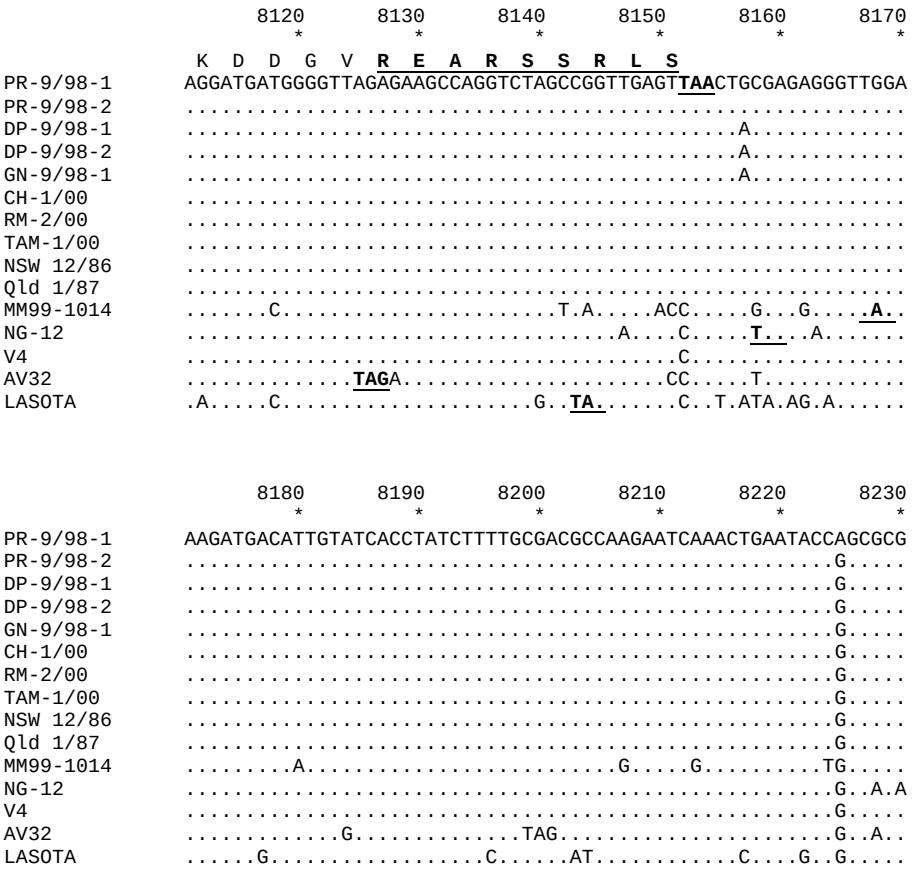

Figure 3. Haemaggutinin-neuraminidase gene sequence comparisons

Notes: Nucleotide sequence differences between isolates are shown while identical nucleotides are indicated by a (.). Numbering is relative to the complete genome sequence of NDV (de Leeuw and Peeters, 1999; Römer-Oberdörfer *et al*., 1999). The terminal amino acid sequence for the HN genes are emboldened as are the various terminator codons for the HN genes. Non-sequenced regions are indicated with an (-).

A phylogenetic analysis (Figure 4) of these sequences revealed a clear relationship to the outbreak viruses as well as to the 'progenitor' virus and two previous Australian isolates, NSW12/86 and Qld1/87. Both latter viruses were shown to have sequences related to the 'progenitor' virus as well as a HN extension of 9 amino acids (REARSSRLS).

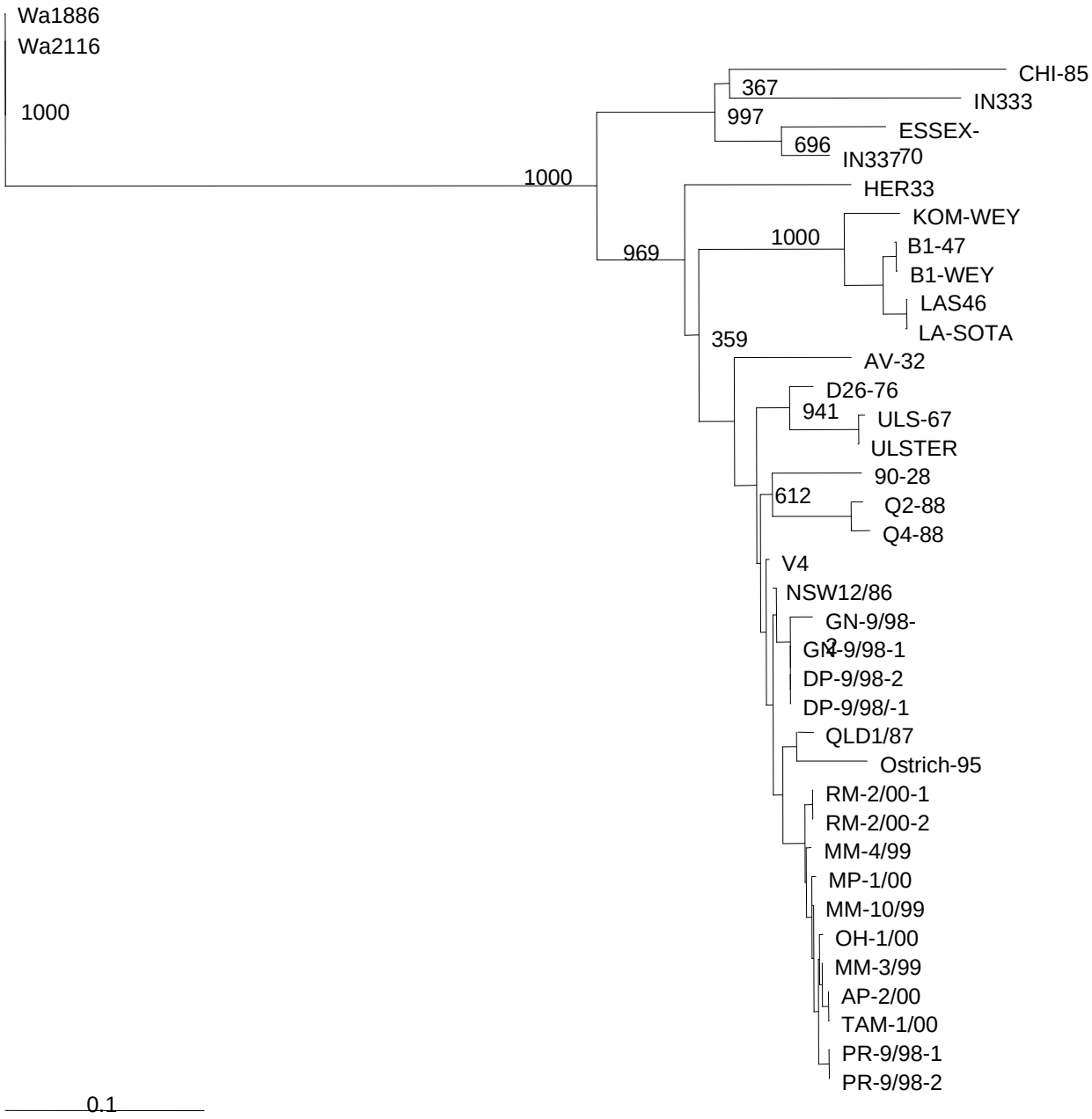

Figure 4. Haemaggutinin-neuraminidase gene phylogenetic analysis

Notes: Phylogenetic analysis of HN C-terminal sequences from bases 7936 to 8304 (Peeters *et al*., 1999; Römer-Oberdörfer *et al*., 1999). The identity of the Australian NDV isolates is given in Materials and Methods, while others are indicated by their GenBank accession numbers. HN sequences are presented as a rooted tree, with NDV wild bird isolates used as the outgroup, after a bootstrap analysis of 1000 replicates was performed.

Sakaguchi *et al.* (1989) showed at least three distinct lineages among 13 strains of NDV isolated over 50 years. These three lineages could be easily grouped by the sizes of the translation products of their HN gene, even though these viruses had steadily accumulated synonymous substitutions. HN size differences correlated well with both nucleotide and amino acid differences and the authors stated that there also appeared to be some correlation with differences in virulence. The latter was not the situation in NDV within Australia as there appears to have been a slow evolution of these viruses from the avirulent isolates identified in the 1960's, to the appearance of respiratory viruses in the late 1980's and finally the emergence of virulent viruses in the 1998-2000 outbreaks. The identification of viruses NSW 12/86 and Qld 1/87 with HN extensions of 9 amino acids, the respiratory condition caused by Peats Ridge virus and, finally, the isolation of fully virulent NDV from the NSW area, all with HN extensions of 9 amino acids, clearly point to the lineage of this NDV outbreak as arising from endemic Australian viruses.

Alexander *et al.* (1986) reported the isolation of two types of antigenically distinct NDV from wild birds in Australia. One group, typified by isolate Wa1886, was quite distinct at the nucleotide and amino acid level from the current outbreak viruses and grouped with previously isolated wild bird isolates. However the other group was shown to be antigenically related to Queensland V4/66 using a panel of HN monoclonal antibodies. This latter group, typified by isolates Wa4317 and Wa4332, have been shown to be distinct from the current outbreak viruses by Western blotting experiments (Della-Porta, unpublished), as they have HN extension of between 24-45 amino acids. Gene sequence analyses of the F_0 and HN C-termini of these ND viruses are currently underway to clarify this issue, but it does raise the possibility that feral birds could be a source of infection with this virulent Australian isolate of NDV. However screening of many wild birds during the recent outbreaks has failed to support this suggestion.

The initial outbreak of virulent NDV at Dean Park appears to have been in progress for up to a month prior to the suspicion of NDV involvement. It is interesting that both the F and HN gene sequence analyses place the Peats Ridge and subsequent virulent and avirulent viruses as being more closely related to each other than the Dean Park viruses. Complete genome nucleotide sequence analyses (to be reported in the next section) of progenitor viruses and virulent viruses support this observation.

Whole genome analysis

The complete genomes of ten NDV isolates from the 1998-2000 outbreaks as well as a 2001 isolate of the progenitor virus and the virus responsible for the Meredith (Victorian) 2002 outbreak have been completely sequenced to give a database for genetic drift of the virus in Australia.

Gene sequence comparisons of the initial outbreak viruses from Dean Park with those from subsequent outbreaks at Mangrove Mountain, have demonstrated that the fusion, nucleocapsid and L (RNA dependant-RNA polymerase) genes of the Mangrove Mountain isolates were five times less variable at the nucleotide and amino acid level than were the matrix, phosphoprotein or HN genes (Figures 5, 6 and 7). Importantly, analysis of the mutation rate of the entire fusion gene compared to that of the fusion cleavage site (Figure 7), showed that there did not exist a hot spot for mutation at the latter site which would have been responsible for the emergence of virulent virus from the avirulent progenitor virus. In the comparison of the matrix protein of the Dean Park and Mangrove Mountain outbreak viruses, subtle differences were observed between isolates at the two sites. The matrix protein nuclear localisation signal (KR.KK……RK.RR; Figure 5) for the Dean Park isolates was different from the Mangrove Mountain isolates and the Meredith isolates which had the sequence (K**K**.KK……**G**K.RR). This observation supports the contention that the Dean Park outbreak arose from a slightly different 'progenitor' virus from the other outbreaks seen in Australia and may have been a separate and distinct event, although other explanations are possible.

```
DP 9/98-1      (   1)   MDSSRTIRLYFDSTFPSSNLLAFPIVLQDTGDGKKQIAPQYRIQRLDSWT
MM-3/99        (   1)   ................S...........................

DP 9/98-1      (  51)   DSKEDSVFITTYGFIFQVGNEEATIGMINDNPRRELLSTAMLCLGSVPNV
MM-3/99        (  51)   ....................V.......K.................

DP 9/98-1      ( 101)   GDLVELARACLTMVVTCKKSATNTERMVFSVVQAPQVLQSCRVVANKYSS
MM-3/99        ( 101)   ........................................K........

DP 9/98-1      ( 151)   VNAVKHVKAPEKIPGSGALEYKVNFVSLTVVPRKDVYEIPTAALKVSGSS
MM-3/99        ( 151)   ..................................K............

DP 9/98-1      ( 201)   LYNLALNVTIDVEVNPKSPLVKSLSKSDSGYYANLFLHIGLMSTVDKRGK
MM-3/99        ( 201)   .....F........D................V.............K..

DP 9/98-1      ( 251)   KVTFDKLERKIRRLDLSVGLSDVLGPSVLVKARGARTKLLAPFFSSSGTA
MM-3/99        ( 251)   ........G.......................................

DP 9/98-1      ( 301)   CYPIANASPQVAKILWSQTACLRSVKIIIQAGTQRAVAVTADHEVTSTKL
MM-3/99        ( 301)   ........................V.......................

DP 9/98-1      ( 351)   EKGHTIAKYNLFKK
MM-3/99        ( 351)   ..........P...
```

Figure 5. Comparison of matrix protein sequences for Dean Park isolate 9/98-1 and Mangrove Mountain isolate MM-3/99

Notes: The nuclear localisation signal (amino acids 247-263) is emboldened. Variant amino acids are given below the Dean Park sequence, while identical amino acids are indicated by a (.)

```
DP 9/98-1      (   1)   MSSVFDEYEQLLAAQTRPNGAHGGGEKGSTLKVEVPVFTLNSDDPEDRWN
MM-3/99        (   1)   ............V..................................

DP 9/98-1      (  51)   FAVFCLRIAVSEDANKPLRQGALISLLCSHSQVMRNHVALAGKQNEATLA
MM-3/99        (  51)   .................................................

DP 9/98-1      ( 101)   VLEIDGFTNSVPQFNNRSGVSEERAQRFMMIAGSLPRACSNGTPFVTAGV
MM-3/99        ( 101)   .........G.....................................

DP 9/98-1      ( 151)   EDDAPEDITDTLERILSIQAQVWVTVAKAVTAYETADESETRRINKYMQQ
MM-3/99        ( 151)   ............................M....................

DP 9/98-1      ( 201)   GRVQKKYILHPVCRSAIQLTIRQSLAVRIFLVSELKRGRNTAGGTSTYYN
MM-3/99        ( 201)   .................................................

DP 9/98-1      ( 251)   LVGDVDSYIRNTGLTAFFLTLKYGINTKTSALALSSLSGDIQKMKQLMRL
MM-3/99        ( 251)   .................................................

DP 9/98-1      ( 301)   YRMKGDNAPYMTLLGDSDQMSFAPAEYAQLYSFAMGMASVLDKGTSKYQF
MM-3/99        ( 301)   .................................................

DP 9/98-1      ( 351)   ARDFMSTSFWRLGVEYAQAQGSSINEDMAAELKLTPAARRGLAAAAQRVS
MM-3/99        ( 351)   .................................................

DP 9/98-1      ( 401)   EDTSSMDMPTQQAGVLTGLSDGGSQAPQGALSRSQGQPDTGDGETQFLDL
MM-3/99        ( 401)   ............................N....................

DP 9/98-1      ( 451)   MRAVANSMREAPNSAQGTPQPGPPPTPGPSQDNDTDWGY
MM-3/99        ( 451)   ....................................
```

Figure 6. Comparison of nucleocapsid protein sequences for Dean Park isolate 9/98-1 and Mangrove Mountain isolate MM-3/99

Notes: Variant amino acids are given below the Dean Park sequence, while identical amino acids are indicated by a (.)

```
DP 9/98-1      (   1)   MGFRSSTRIPVPLMLTVRVMLALSCVCPTSALDGRPLAAAGIVVTGDKAV
MM-3/99        (   1)   .........S.....................................

DP 9/98-1      (  51)   NIYTSSQTGSIIIKLLPNMPKDKEACAKAPLEAYNRTLTTLLTPLGDSIR
MM-3/99        (  51)   .................................................

DP 9/98-1      ( 101)   RIQESVTTSGGRRQRRFIGAIIGGVALGVATAAQITAASALIQANQNAAN
MM-3/99        ( 101)   .................................................

DP 9/98-1      ( 151)   ILRLKESIAATNEAVHEVTNGLSQLAVAVGKMQQFVNDQFNKTAQELDCI
MM-3/99        ( 151)   .................................................

DP 9/98-1      ( 201)   KITQQVGVELNLYLTELTTVFGPQITSPALTQLTIQALYNLAGGNMDYLL
MM-3/99        ( 201)   .................................................

DP 9/98-1      ( 251)   TKLGVGNRQLSSLISSGLITGNPILYDSQTQLLGIQVTLPSVGNLNNMRA
MM-3/99        ( 251)   ......S..........................................

DP 9/98-1      ( 301)   TYLETLSVSTTKGFASALVPKVVTQVGSVIEELDTSYCIETDLDLYCTRI
MM-3/99        ( 301)   .................................................

DP 9/98-1      ( 351)   VTFPMSPGIYSCLSGNTSACMYSKTEGALTTPYMTLKGSVIANCKMTTCR
MM-3/99        ( 351)   .................................................

DP 9/98-1      ( 401)   CADPPGIISQNYGEAVSLIDRQSCNILSLDGITLRLSGEFDATYQKNISI
MM-3/99        ( 401)   .................................................

DP 9/98-1      ( 451)   LDSQVIVTGNLDISTELGNVNNSISNALDKLEESNNKLDKVNVKLTSTSA
MM-3/99        ( 451)   Q..............................S.........A....

DP 9/98-1      ( 501)   LITYIVLTVISLVCGILSLVLACYLMYKQKAQQKTLLWLGNNTLDQMRAT
MM-3/99        ( 501)   .................................................

DP 9/98-1      ( 551)   TKM
MM-3/99        ( 551)   ...
```

Figure 7. Comparison of fusion protein sequences for Dean Park isolate 9/98-1 and Mangrove Mountain isolate MM-3/99

Notes: The fusion cleavage signal (amino acids 112-117) is emboldened. Variant amino acids are given below the Dean Park 1998 sequence, while identical amino acids are indicated by a (.)

Nucleotide sequence variation of the 'progenitor' virus and virulent Australian NDV with time (1998-2002)

To obtain some idea as to how fast the nucleotide sequence of the NDV 'progenitor' virus was mutating in the field with time, the complete genome of several isolates of this avirulent NDV were sequenced and compared. The viruses chosen for this study were the original 'progenitor' virus isolated from Peats Ridge, Mangrove Mountain, in 1998, a 'progenitor' virus isolated in NSW in 1999, and a 'progenitor' virus isolated in NSW in 2001.

Nucleotide sequence comparisons of the complete genomes with time (Figure 8) showed a slow accumulation of nucleotide changes in the 'progenitor' virus genome. From 1998 to 1999 the accumulated errors were 0.52% of the genome, while that observed for the 2001 virus increased to 0.94%. Comparison of the 1999 and 2001 isolates showed a rate of nucleotide variation of 0.67%. Most of these nucleotide changes occurred in the third base position of a codon and thus did not alter the translation products derived from the NDV genes. Comparison of the fusion nucleotide and protein sequences showed that, from the 1998 to 1999, only two amino acids were altered at positions 5 and 257 (Phe→Ser and Asn→Ser) while there were also two amino acid changes in the 2001 isolate at positions 453 and 509 (Ser→Phe and Val→Ile) when compared to the original isolate. Amino acid rates of change for these were thus lower than that observed at the nucleotide level and were at approximately 0.3%, with most changes involving conservative amino acid substitutions (Table 1).

Similar rates of change were observed when virulent NDV isolates were compared from 1998, 1999 and 2002. From 1998 to 1999 the observed rate of nucleotide change was 0.50% while that seen from 1998 to 2002 was 1.12%. Thus it would appear that a slow yearly rate of nucleotide change of approximately 0.2-0.25% had occurred, with the amino acid yearly rate of change being 0.18%. There appeared to be no increased rate of change at the fusion cleavage site when compared to other regions of the genome which would have explained the appearance of virulent virus from the circulating avirulent NDVs in Australia.

Thus the nucleotide sequence of both these virulent and avirulent viruses were exhibiting a slow rate of change with time, as expected for an RNA virus. This property of error rate is easily explained by the fact that the RNA dependant-RNA polymerase of NDV has no editing or error correction mechanism inherent in its enzymatic function. Thus the genome can rapidly accumulate errors in its genome during replication. This accumulation of errors in individual genomes then leads to the situation whereby many slightly different genomes compete for their ability to replicate in an infected cell, leading to what is termed a quasi-species. The fitness of this quasi-species for continued propagation both *in vivo* and *in vitro* is defined by Darwinian selection pressures on the replicating virus. This leads to the situation whereby when we determine the nucleotide sequence of a field isolate, we are actually determining the consensus sequence of the quasi-species. Thus to obtain a better understanding of the dynamics behind the observed rates of change in the nucleotide sequence of these field isolates it was decided to investigate the rate of change of a plaque-purified virus (i.e. a virus with a defined nucleotide sequence with few nucleotide changes) both *in vivo* and *in vitro.*

4.2 Quasi-species analysis of fusion gene sequences

To perform the quasi-species analysis of the individual sequences that comprised the population of viruses in a field isolate, a proof-reading Taq polymerase (Pfu polymerase) was used to amplify a 525 nucleotide region of the NDV genome (from nucleotides 4711-5236) which was either cloned directly into a Topo1 cloning vector (Promega) or digested with Dde1 and cloned into a Sma1 digested-CIP pUC 19 vector. Individual clones containing this DNA sequence were then sequenced, and the individual sequences comprising the quasi-species population compared to the consensus sequence derived for the field isolate. From this analysis it was determined that approximately 50-60% of the individual sequences within the viral population has sequences identical to the consensus sequence. However the remaining individuals had single or multiple nucleotide changes in their sequences. These differences resulted in amino acid changes in the F protein if they occurred in either the first or second nucleotide positions of an amino acid codon. A more detailed analysis of these changes is given in Tables 2 and 3. However, in the analysis of these changes only in one isolate (PR32) were any significant alterations seen in the fusion cleavage site (Table 3)? In this field isolate it was demonstrated that, although the consensus sequence for the field isolate was avirulent, having an F cleavage sequence of RRQGRF, the population that made up the consensus sequence had 26% of the individuals present with a virulent F cleavage sequence of RRQRRF. From this population of viruses, plaque purified isolates were made of viruses containing only virulent or avirulent F cleavage sequences (see later). Both the field isolate and the virulent and avirulent plaque-purified viruses had an ICPI of 1.8 compared to the 98-1154 progenitor virus which had an ICPI of 0.6 (the same as determined for the V4 vaccine strain of NDV).

The other NDV isolates examined did not show the presence of any other virulent sequences in their quasi-species populations. Thus, from the number of individuals sequenced within the quasi-species comprising the isolate 98-1154, it was possible to place a limit of less than 1 in every 140 viruses could have a virulent sequence. It was decided to apply a more sensitive procedure (Real-Time PCR) to detect virulent NDV sequences which could be present in the field isolates of 98-1154 progenitor virus. PCR reactions were set up utilising primers which could detect the presence of the terminal F cleavage amino acid sequences of GRL (avirulent; Figure 9) or RRF (virulent; Figure 9) or LSSP (control sequence). From these analyses it was possible to detect the presence of virulent cleavage site sequences in the avirulent population of sequences at a level of 1 in 1,000 to 1 in 5,000. Although

these virulent sequences were present in the viral quasi-species they generally did not give the symptoms of a virulent virus in infected poultry, even when the level of virulent virus was as high as 1 in 4 (as demonstrated with the PR32 field isolate). Thus, there must be some other mechanism operating to cause virulent virus to dominate the quasi-species.

```
98-1154         CCATCTCCTTATGTGACCCAAGGGGGCGAAATGGCACTCAATAAACTCTCGCAACCGGTG
99-0868-hi      CCATCTCCTTATGTGACCCAAGGGGGCGAAATGGCACTCAATAAACTCTCGCAACCGGTG
01-1108         CCATCTCCTTATGTGACCCAAGGGGGTGAAATGGCACTCAATAAACTCTCGCAACCGGTG
                ************************** **********************************

98-1154         CAACGCCCCTATGAATTGATTAAACCCGCCACGGCAAGCGGGCCAGATATAGGAGTGGAG
99-0868-hi      CAACGCCCCTCTGAATTGATTAAACCCGCCACGGCAAGCGGGCCAGATATAGGAGTGGAG
01-1108         CAACGCCCCTCTGAATTGATTAAACTCGCCACGGCAAGCGGGCCAGATATAGGAGTGGAG
                ********** ************** **********************************

98-1154         AAAGACACTGTCCGTGCATTGATCATGTCACGCCCTATGCATCCGAGCTCTTCAGCTAGG
99-0868-hi      AAAGACACTGTCCGTGCATTGATCATGTCACGCCCTATGCATCCGAGCTCTTCAGCTAGG
01-1108         AAAGACACTGTCCGTGCATTGATCATGTCACGCCCTATGCATCCGAGCTCTTCAGCTAGG
                ************************************************************

98-1154         CTCTTGAGCAAACTGGACGCAGCCGGATCGATTGAGGAAATCAGAAAAATCAAGCGCCTT
99-0868-hi      CTCTTGAACAAACTGGACGCAGCCGGATCGATTGAGGAAATCAGAAAAATCAAGCGCCTT
01-1108         CTCCTGAGCAAACTGGACGCAGCCGGATCGATTGAGGAAATCAGAAAAATCAAGCGCCTT
                *** *** **************************************************

98-1154         GCACTGAATGGCTAATCACCACCGCGACCCGCAGCAGATCCCTGTCCACCCAGCACCACA
99-0868-hi      GCACTGAATGGCTAATCACCACCGCGACCCGCAGCAGATCCCTGTCCACCCAGCACCACA
01-1108         GCACTGAATGGCTAATCACCACCGCGACCCGCAGCAGATCCCTGTCCACCTAGCACCACA
                ************************************************* *********

98-1154         CGGTATCTGCACCAAGCTCCTCTCTGCAAATCCAAGGTCCAACACCCCGAGCGACAACCC
99-0868-hi      CGGTATCTGCACCAAGCTCCTCTCTGCAAACCCAAGGTCCAACACCCCGAGCGACAACCC
01-1108         CGGTATCTGCACCAAGCTCCTCTCTGCAAACCCAAGGTCCAACACCCCGAGCGACAACCC
                ****************************** ******************************

98-1154         TGTCCTGCTTCCTCTGTCCCACCAAATGATCGCGCAGCTGCAATCAATTCAGCTATATTA
99-0868-hi      TGTCCTGCTTCCTCTGTCCCACCAAATGATCGCGCAGCTGCAATCAATTCAGCTATATTA
01-1108         TGTCCTGCTTCCTCTGTCCCACCAAATGATCGCGCAGCTGCAATCAATTCAGCTATATTA
                ************************************************************

98-1154         AGGATTAAGAAAAAATACGGGTAGAATCGGAGTGCCCCGATTGTGTCAAGATGGACTCAT
99-0868-hi      AGGATTAAGAAAAAATACGGGTAGAATCGGAGTGCCCCGATTGTGCCAAGATGGACTCAT
01-1108         AGGATTAAGAAAAAATACGGGTAGAATCGGAGTGCCCCGATTGTGCCAAGATGGACTCAT
                ******************************************* **************

98-1154         CTAGGACAATCAGGCTGTACTTTGATTCTACCTTTCCTTCTAGCAACCTGCTAGCATTCC
99-0868-hi      CTAGGACAATCAGGCTGTACTTTGATTCTACCTTTCCTTCTAGCAACCTGCTAGCATTCC
01-1108         CTAGGACAATCAAGCTGTACTTTGATTCTACCTTTCCTTCTAGCAACCTGCTAGCATTCC
                ************ ***********************************************

98-1154         CGATAGTCCTACAAGACACAGGGGACGGGAAGAAGCAAATCGCCCCGCAATACAGGATCC
99-0868-hi      CGATAGTCCTACAAGACACAGGGGACGGGAAGAAGCAAATCGCCCCGCAATACAGGATCC
01-1108         CGATAGTCCTACAAGACACAGGGGACGGGAAGAAGCAAATCGCCCCGCAATACAGGATCC
                ************************************************************

98-1154         AGCGTCTTGACTCGTGGACAGACAGCAAAGAAGACTCGGTATTCATCACCACCTATGGAT
99-0868-hi      AGCGTCTTGACTCGTGGACAGACAGCAAAGAAGACTCGGTATTCATCACCACCTATGGAT
01-1108         AGCGTCTTGACTCGTGGACAGACAGCAAAGAAGACTCGGTATTCATCACCACCTATGGAT
                ************************************************************

98-1154         TCATCTTTCAGGTTGGGAATGAAGAAGCCACTGTCGGCATGATCAATGATAATCCCAAGC
99-0868-hi      TCATCTTTCAGGTTGGGAATGAAGAAGCCACTGTCGGCATGATCAATGATAATCCCAAGC
01-1108         TCATCTTTCAGGTTGGGAATGAAGAAGCCACTGTCGGCATGATCAATGATAATCCCAAGC
                ************************************************************

98-1154         GCGAGTTACTTTCCACTGCCATGCTATGCCTAGGGAGTGTACCAAATGTCGGAGATCTTG
99-0868-hi      GCGAGTTACTTTCCACTGCCATGCTATGCCTAGGGAGTGTACCAAATGTCGGAGATCTTG
01-1108         GCGAGTTACTTTCCACTGCCATGCTATGCCTAGGGAGTGTACCAAATGTCGGAGATCTTG
                ************************************************************
```

Figure 8. Comparison of nucleotide sequences for progenitor viruses Dean Park isolate 9/98-1 and Mangrove Mountain isolate MM-99/0868HI and NSW isolate 01-1108

Table 1. Variation in nucleotide sequence between NDV isolates

BPPosn	98-1252[1]	98-1154[2]	99-0655 [3]	HI-HA[4]	LO-HA[5]	98-1249[6]	AA change	Comment	Change [Y/N
8067	C	**A**	C	C	C	C	Leu	3rd base change	N
8070	T	**C**	-	-	-	T	Phe	3rd base change	N
8078	T	**A**	-	-	-	T	Y to F	Conservative	Y
8091	T	**C**	-	-	-	T	Pro	3rd base change	N
8100	T	**A**	-	-	-	T	Val	3rd base change	N
8157	A	**G**	-	-	-	A	-	Non coding	N
8225	G	**A**	G	G	G	G	-	Non coding	N
8235	C	**T**	-	-	-	C	-	Non coding	N
8244	C	**T**	-	-	-	C	-	Non coding	N
8310	A	**G**	-	-	-		-	Non coding	N
8365	G	**A**	-	-	-		-	Non coding	N
8393	A	**G**	-	-	-		G to S		Y
8414	C	**A+C**	C	C	C	C	Q to K		Y
8416	A	**G**	-	-	-	-	-	3rd base change	N
8431	G	**A**	-	-	-	-	-	3rd base change	N
8491	A	**G**		-	-		-	3rd base change	N
8530	-	**T**		C	C		-	3rd base change	N
8565	G	**A**		-	-		-	3rd base change	N
8640	-	**C**		T	T		T to I		Y
8737	C	**T**		-	-		-	3rd base change	N
8855	C	**T**		-	-		-	3rd base change	N
8863	A	**G**		-	-		-	3rd base change	N

Table 2. Nucleotide changes, represented as percentage values, detected across 580bp PCR products (spanning the F_0 cleavage motif) generated from individual bacterial colonies using *pfu* DNA polymerase (Promega)

	A Transitions				**T** Transitions				**G** Transitions				**C** Transitions				Nucleotide changes	Fragments sequenced	Other Changes
	T	**G**	**C**	All	**A**	**G**	**C**	All	**A**	**T**	**C**	All	**A**	**T**	**G**	All			
98-1154	9 7.9	38 33.3	2 1.8	49 43.0	14 12.3	-	20 17.5	34 29.8	9 7.9	4 3.5	-	13 11.4		12 10.5	4 3.5	16 14.0	114	142	- G (2) **1.8**
2609-4	5 5.4	37 40.2	4 4.3	46 50.0	7 7.6	1 1.1	18 19.6	26 28.3	4 4.3	3 3.3	1 1.1	8 8.7		10 10.9		10 10.9	92	106	- G (1) - C (1) **2.1**
PR-32	3 6.3	11 23.0	1 2.1	15 31.2	3 6.3	2 4.2	11 23.0	16 33.3	12 25.0	1 2.1	-	13 27.1		1 2.1	1 2.1	2 4.2	48	50	- T (1) - C (1) **4.2**
2609-3	-	18 42.9	3 7.1	21 50.0	1 2.4	-	6 14.3	7 16.7	8 19.0	1 2.4	-	9 21.4		4 9.5		4 9.5	42	53	- G (1) **2.4**
99-0655	6 12.0	19 38.0	-	25 50.0	6 12.0	1 2.0	6 12.0	13 26.0	2 4.0	3 6.0	-	5 10.0		5 10.0	2 4.0	7 14.0	50	50	-
Total Values	23 6.7	123 35.5	10 2.9	**156** **45.1**	31 9.0	4 1.1	61 17.6	**96** **27.7**	35 10.1	12 3.5	1 0.3	**48** **13.9**	0 0	32 9.3	7 2.0	**39** **11.3**	**346**	**401**	7 **2.0**

Table 3. Amino acid changes, represented as percentage values, detected across 580bp PCR products (spanning the F_0 cleavage motif) generated from individual bacterial colonies using *pfu* DNA polymerase (Promega)

NDV isolate	F_0 cleavage motif	Position of nucleotide changes			Nucleotide changes per fragment		Number of changes per fragment				Amino acid changes	Nucleotide changes in the F_0 cleavage sequence	Amino acid changes in the F_0 cleavage site
		1st	**2nd**	**3rd**	**0**	**≥1**	**1**	**2**	**3**	**4**			
98-1154	RRQGRL	27 23.7	48 42.1	39 34.2	71 50.0	71 50.0	39 27.5	22 15.5	9 6.3	1 0.7	81	1 0.7	1 0.7
2609-4	RRQGRL	27 29.3	31 33.7	34 37.0	41 38.7	65 61.3	42 39.6	19 17.9	4 3.8	0 0	55	4 3.8	2 1.9
PR-32	**RRQGRF**	21 43.8	13 27.1	14 29.1	16 32.0	34 68.0	23 46.0	8 16.0	3 6.0	0 0	32	13 26.0	13 26.0
2609-3	RRQRRL	17 40.5	10 23.8	15 35.7	27 51.0	26 49.0	16 30.2	5 9.4	4 7.5	1 1.9	27	2 3.8	2 3.8
99-0655	RRQRRF	14 28.0	19 38.0	17 34.0	16 32.0	34 68.0	21 42.0	11 22.0	1 2.0	1 2.0	34	2 4.0	2 4.0

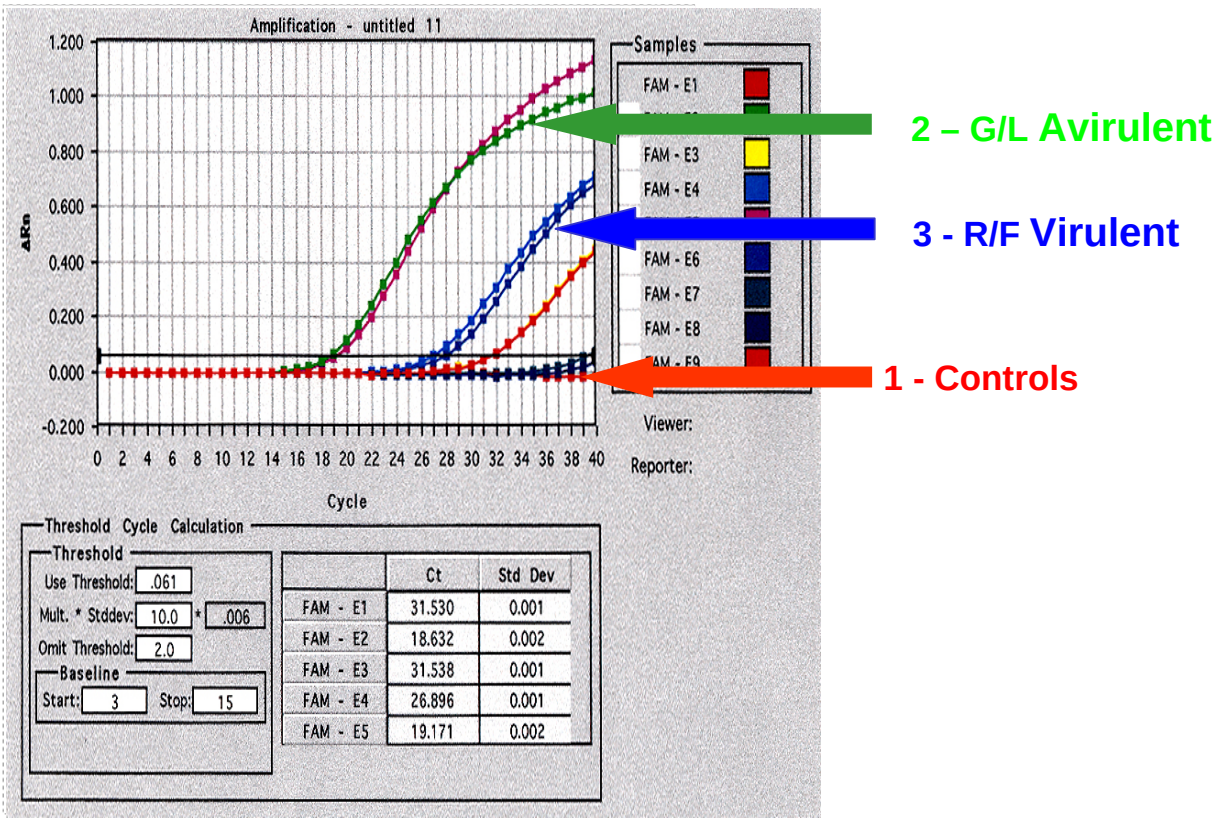

Figure 9. Real-Time PCR analysis of progenitor virus field isolate 98-1154

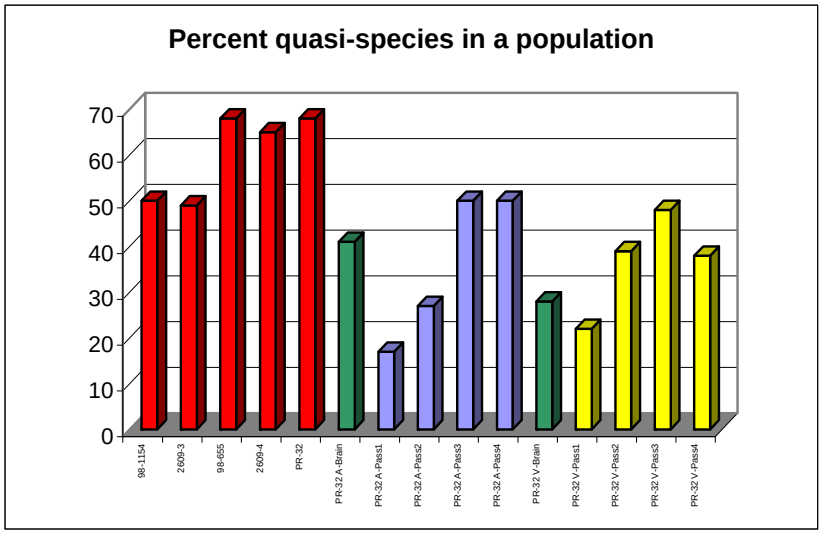

Figure 10. Passage of plaque-purified PR32 in chickens and tissue culture

The percent quasi-species present in the total viral population of a field isolate is shown on the Y axis of the figure, while each isolate examined is indicated on the X axis of the figure.

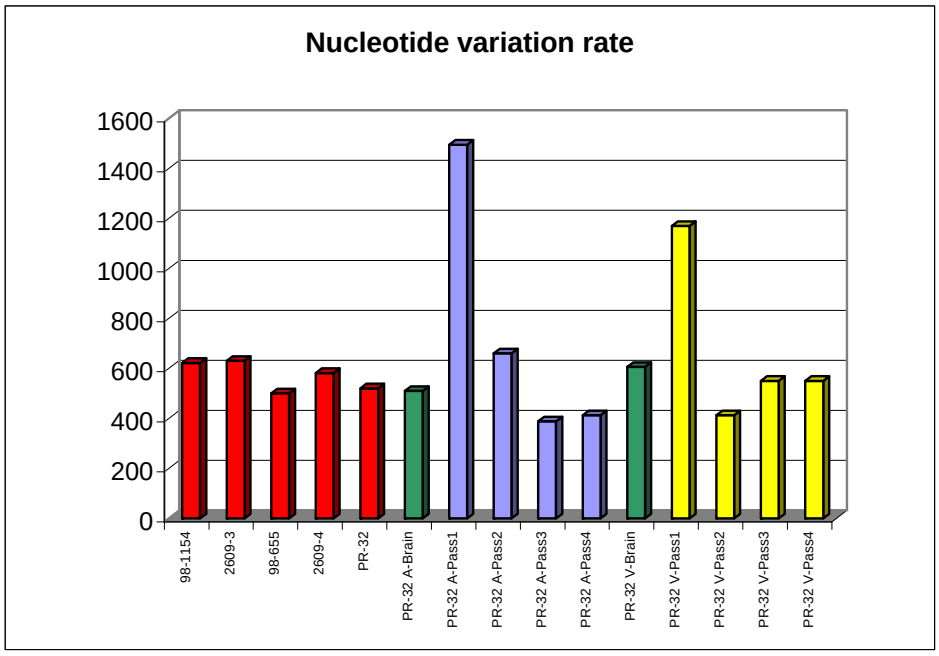

Figure 11. Nucleotide variation rate with serial passage

The field isolates examined are indicated on the X axis of the figure while the observed transcriptional error rate is expressed as a ratio of the total number of observed nucleotide changes per nucleotides sequenced for a particular field isolate and are shown on the Y axis of the figure (nucleotide change per total nucleotides sequenced).

From the experiments represented in Figures 10 and 11 it was demonstrated that the field isolates 98-1154, 2609-3, 98-655 and PR32 had quasi-species levels of between approximately 50-60% and a nucleotide error rate of approximately 1 in every 500-600 nucleotides. However plaque-purified PR32 (virulent and avirulent), when passaged once in birds, had quasi-species levels of 25-38% and nucleotide error rates approaching that of field isolates of between 1 in every 500-600 nucleotides. When passaged *in vitro,* plaque-purified viruses gradually approached the level of quasi-species and nucleotide error rates seen in field isolates after two to three passages. Therefore, plaque-purified viruses very quickly approach the variation seen in field isolates within a short number of passages either *in vivo* or *in vitro*. However the overall consensus sequence observed in either virulent or avirulent field isolates was generally maintained over longer time periods of several years, with only a small (0.2-0.25% *per annum*) drift in consensus sequences observed.

4.3 Rescue of virulent virus from a quasi-species having an avirulent consensus sequence

The previous analyses of variability within quasi-species of NDV demonstrated that viruses with virulent F cleavage sequences could be harboured at low levels in field isolates having an avirulent F cleavage sequence. Normally, avirulent NDV are not able to undergo lytic infection in tissue culture unless the protease trypsin is added to the culture media. However virulent viruses have no such requirement. This property lies entirely within the F cleavage site, as only F proteins with the sequence RRQRRF are able to be cleaved by the ubiquitous proteases of tissue culture cells; those with RRQGRL need a trypsin like protease to cleave them. Experiments were done in tissue culture to attempt to amplify the low levels of endogenous virulent virus up from the avirulent background by prolonged tissue culture. Normally, within two days of infecting tissue culture cells with a virulent virus, altered cell morphology in syncitial cell formation (cell fusion) is observed.

A stock of the avirulent 'progenitor' virus (98-1154) with a low level of virulent virus present in the viral population (approximately 1:1,000) was inoculated on chicken embryo fibroblast cells in the absence of added trypsin. After seven to eight days, syncitia were seen to form in tissue culture and the virus released from the cells were harvested and a quasi-species analysis performed. The results showed that the level of virulent virus in the tissue culture had risen to 18% of the total observed.

There are several other clades of Australian viruses which have the same properties as that associated with the 'progenitor' virus. These isolates have the F cleavage sequence of RKQGRL and an HN extension of 7 amino acids. To date, there have been 12 such isolates made from both NSW and Queensland. Four of these isolates (Q2-88, Q4-88, NG4 and NG2) were propagated in CEF cells in the absence of exogenous trypsin. After four days, syncitial formation occurred and quasi-species analysis of the released virus from tissue culture cells demonstrated virulent F cleavage sequences were present in 1-2% of the viral population. This observation demonstrated that there were other Australian viruses that were also two nucleotides away from having a virulent F cleavage motif and had the potential to cause a virulent NDV outbreak.

The above experiments demonstrated that a virulent virus could be maintained in a number of different isolates of NDV and not just the 'progenitor' class of virus, and that these virulent viruses would remain in the genetic background until a selection pressure was placed upon the quasi-species such that the virulent virus had a selected replicative advantage and became the dominant sequence in the quasi-species.

4.4 The ability of other avian diseases to alter the course of NDV infections

In many instances, field viral isolates from infected poultry demonstrated the presence of other avian viruses as well as NDV. In particular, the coronavirus, infectious bronchitis virus (IBV) was often co-isolated along with NDV in infected poultry. Therefore, the possibility that a pre-existing viral infection could alter the tissue trophism of NDV was investigated by infecting birds with plaque purified NDV isolate PR-32, after infection with either IBV (which replicates in the respiratory tract – the same location as PR32 replication) or infectious bursal disease virus (IBDV; an immuno-suppressive virus).

IBV infection

SPF birds at 21 days-of-age were inoculated with the IBV S strain (Websters live virus vaccine strain) by the application of one drop via the ocular route (equivalent to one effective dose of the virus, according to the manufacturer's instructions). Seven days later, birds were challenged with plaque-purified avirulent NDV (isolate PR32) and monitored for the development of symptoms. Birds were also challenged with three other doses of PR32 in which the avirulent virus was diluted with plaque-purified avirulent NDV (98-1154) in the ratios of 1:4, 1:100 and 1:1,000. For each group of six birds (three per cage), control birds were also set up which were not pre-vaccinated with IBV (see Figure 12 for a schematic for the arrangement of birds).

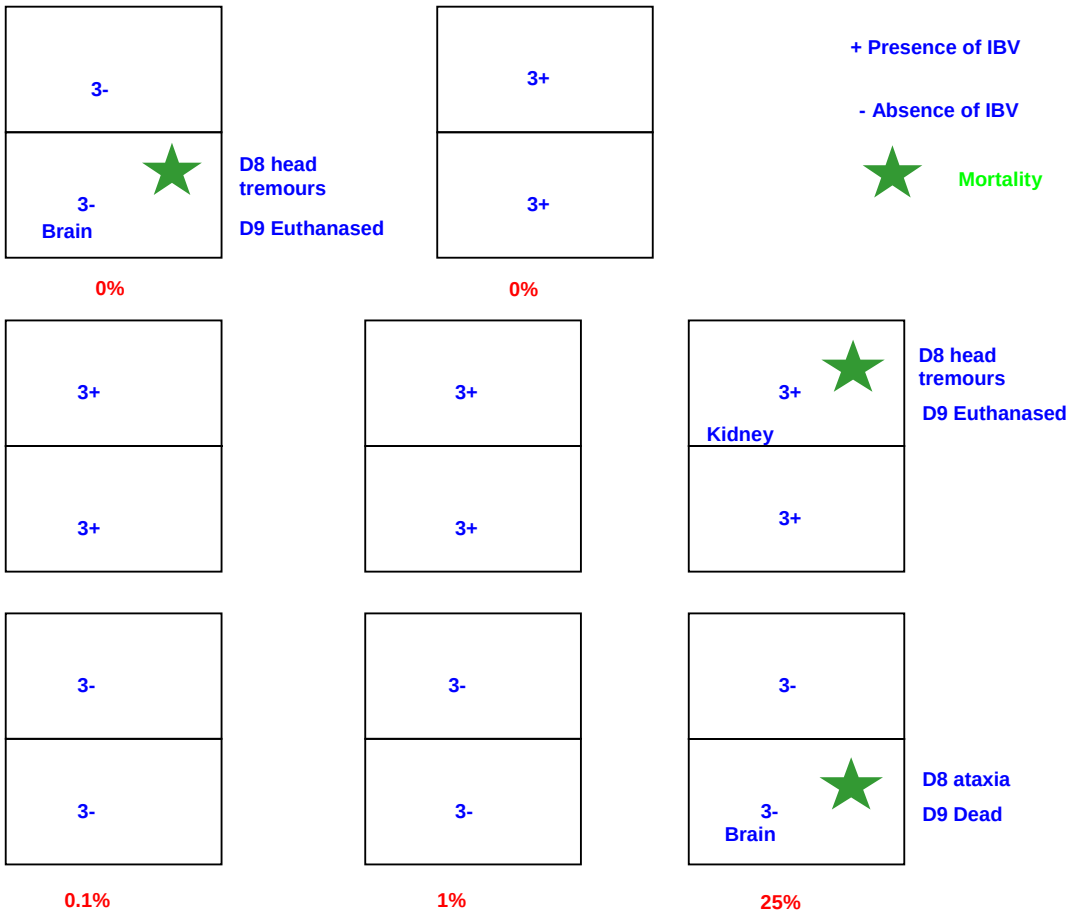

Figure 12. Challenge of IBV infected birds with NDV

Eight days after inoculation with NDV, three birds (one from a group which were inoculated solely with undiluted PR32, one from the group inoculated with IBV and then PR32:98-1154 at a ratio of 1:4 and one from the group inoculated solely with PR32:98-1154 at a ratio of 1:4) developed nervous symptoms and died or were euthanased on day nine after NDV inoculation. Tissue samples were harvested from the three birds above, cultured in eggs and allantoic fluids analysed by PCR for the presence of NDV. Sequence analyses were done on amplified DNA and showed the presence of virulent virus in the brain and kidney of these birds. The sequence determined from these tissues matched that of PR32 and not 98-1154 (results not shown). The neural signs showed by the three birds in this experiment were of interest in that they were transitory. With the exception of the bird that died, the other two birds recovered completely within 24 hours and could not be distinguished from any other birds in the flock. This had been observed with other birds infected with virulent NDV from the Australian outbreaks and may be a factor in the ability to clearly diagnose the symptoms associated with these viral infections. All other birds in the experiment exhibited no signs of either IBV or NDV infection (either respiratory or neural). PCR amplification of tissues and cloacal swabs from these birds were negative for IBV or NDV.

IBDV infection

Three week old SPF birds were inoculated with the vaccine strain of IBDV (IBDV Strain V877); 50 µl/eye, according to the manufacturer's instructions) and observed for any clinical signs due to immunisation. None were apparent. Seven days after immunisation, birds were inoculated with PR32 (at a ratio of 1:10 with PR32:98.1154 plaque-purified virus) via the ocular route. Two days after NDV exposure the first signs of NDV became apparent and three days after NDV exposure one third of the flock was exhibiting respiratory symptoms. On the fourth day after NDV exposure the entire flock (of 26 birds) was exhibiting respiratory symptoms, with eight also exhibiting neural signs. A more detailed description of the experiment is shown in Table 4.

Table 4a. Sequale of IBDV-NDV infection of poultry

4/9/02	Challenged with PR32 (plaque 4.2) diluted to a ratio of 1:10 of Peats Ridge virus
5/9/02	All birds healthy, no clinical signs
6/9/02	All birds healthy, no clinical signs
7/9/02	1 sick (swollen eyes, depressed)
8/9/02	10 sick (swollen eyes and heads, depressed, gasping breathing)
9/9/02	1 dead, rest sick to severely sick (swollen eyes and heads, depressed, not eating, diarrhea, some with gasping breathing)
10/9/02	1 dead, rest sick to severely sick (swollen eyes and heads, depressed, not eating, diarrhea, some with gasping breathing, tracheal exudates, froth in mouth). Some recovering.
11/9 to 18/9	See Table 4b below
18/9/02	All birds euthanased and brains collected for histology and PCR

Table 4b. Sequale of IBDV-NDV infection of poultry

Date	Total Alive	Normal	Respiratory	Nervous	Both	Dead	Total Dead
5/9	25	25					
6/9	25	25					
7/9	25	24	1				
8/9	25	15	10				
9/9	24		24#	(8???)		1	1
10/9	23		23			1	2
11/9	22	11	3*	7	1	1(NT)	3
12/9	21	11	2*	6	2	1(47)	4
13/9	18	9	2*	6	1	3(NT,49,96)	7
14/9	16					2(97,98)	9
15/9	14					2(NT,48)	11
16/9	12	4	1	7@	-	2(2xNT)	13
17/9	11	4(46)	-	7(50)	-	-	14
18/9	11	4(46)	-	7(50)	-	-	14

Notes:

8 birds tagged (#'s 46, 47, 48, 49, 50, 96, 97, 98) showing possible nervous signs on 9/9. Other birds were left untagged but monitored closely for clinical signs.

* tracheal exudates, froth in mouth

@ 1 bird (NT) euthanased as it was paralysed

NT not tagged

Table 4c. Sequale of IBDV-NDV infection of poultry – clinical signs

Clinical signs in birds at termination of the experiment	
Bird # 46	Normal?
Bird #50	Severe head/neck tremors
Bird # no tag 1	Fine tremors
Bird # no tag 2	Fine tremors, gasping
Bird # no tag 3	Depressed, hydroencephaly
Bird # no tag 4	Fine tremors
Bird # no tag 5	Star gazing
Bird # no tag 6	Star gazing
Bird # no tag 7	Normal
Bird # no tag 8	Normal
Bird # no tag 9	Normal

Brain tissues from untagged birds 1-9 and tagged birds 46, 47, 49, 50 and 96 were examined histologically and via immunoperoxidase staining for NDV antigen. Only birds 49, 50 and 96 showed evidence of encephalitis in the brain while only birds 47 and 49 were positive by immunoperoxidase staining. PCR analysis showed that 2, 3, 5, 8, 47 and 49 were positive for virulent virus, and virus isolation from the brains of birds 47 and 49 was successful. Gene sequence analysis of PCR products and rescued virus showed identical sequences with a deduced virulent fusion cleavage protein sequence of RRQRRF.

5. Discussion

Gene sequence analysis of Australian NDV isolates has proven to be a powerful tool to elucidate the diversity of NDV strains circulating in the poultry industry. Retrospective analysis also proved invaluable for tracing the lineage of viruses involved in the outbreaks of virulent NDV in Australia. Phylogenetic analyses showed that the presence of an avirulent 'precursor' virus that evolved from a pre-existing Australian clade of viruses having a 9 amino extension at the C-terminus of the HN gene, was intimately involved with the NDV outbreaks in NSW and Victoria (1998-2002). The existence of this clade of viruses could be traced to Mangrove Mountain as early as 1991, prior to the emergence of virulent NDV in Australia.

Ten complete genomes of virulent and avirulent NDV isolates from the 1998-2002 outbreaks were sequenced to show the slow genetic drift of this virus over this time span and provide a database for future investigations. Genetic analysis of these viruses showed that the fusion gene, and in particular the fusion cleavage signal, was no more susceptible to genetic mutation than other regions of the genome.

Quasi-species analysis showed that plaque-purified virus was able to quickly generate variant genomes, which had the same level of variability as seen in field isolates, within two to three passages *in vivo* or *in vitro.* Quasi-species analysis also showed that some field isolates were able to accumulate high levels of virulent virus in their populations without this being overtly apparent in the symptoms exhibited by infected poultry. Real time PCR also demonstrated that virulent virus could be present in very low levels in field isolates but that this virus remained hidden and undetected until a selection pressure was exerted upon the quasi-species. Such a selection pressure could be mimicked using tissue culture breakthrough experiments wherein avirulent virus cultured in CEF cells eventually revealed the presence of virulent virus in its quasi-species which, from one passage, could increase from undetectable levels to 18% of the final population. Tissue culture breakthrough experiments also revealed the presence of virulent virus present in respiratory NDV from another clade of viruses characterised by having an HN extension of 7 amino acids.

Experiments to test the ability of other avian viral infections to apply the selection pressure, ameliorate the symptoms of NDV infection and allow emergence of virulent virus, provided some interesting avenues for further investigation. Immunisation of birds with IBV prior to NDV infection did not alter the subsequent course of NDV symptoms in these birds. On the other hand, prior immunisation with IBDV did appear to have an effect. Within a few days the respiratory symptoms normally associated with infection with a NDV having a 9 amino extension to the HN gene, became apparent in the whole flock. Within five days after NDV infection, classic symptoms of neural involvement were seen in approximately one third of the flock. PCR and histological analysis showed the presence of virulent NDV in the brains of these birds. While these results must be interpreted with some caution, as these experiments were preliminary only and there was no control (NDV challenged only) group, they suggest that IBDV infection or immunosuppression may be one trigger for the emergence of virulent NDV from avirulent NDV quasi-species.

6. Implications

Australia has several clades of NDV virus that are two nucleotides from virulence as defined by their F gene cleavage motifs. These viruses are endemic within Australia and viruses from one of these clades were responsible for the emergence of virulent NDV in Australia. Vaccination with the avirulent V4 strain of virus is capable of immunising poultry and protecting against disease caused by the virulent virus from these outbreaks and is an effective protective procedure. Quarantine and slaughter alone were not effective in controlling NDV outbreaks in NSW until they were combined with the vaccination program.

Effective diagnostic procedures are available to detect the presence of the progenitor virus in Australia and thus it would be possible to survey poultry flocks for the presence of this virus and eradicate it via culling and vaccination.

The implication of an involvement of immunosuppression in the emergence of virulent NDV and the possibility that other avian viruses may exert an influence on the host is worthy of further investigation.

7. Recommendations

Surveillance and vaccination against Australian NDV isolates capable of generating virulent NDV in the poultry industry should ensure that NDV outbreaks are minimised if not eradicated. However this level of surveillance would require a large degree of commitment on the part of industry and other growers.

The possibility that infection with IBDV and/or other immunosuppressive agents may promote the emergence of virulent NDV should be further investigated.

8. References

Albiston, H.E. and Gorrie, C.J. (1942). Newcastle disease in Victoria. *Aust .Vet. J.* **18**: 75-79.

Collins, M.S., Bashiruddin, J.B. and Alexander, D.J. (1993). Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch. Virol.* **128**: 363-370.

Gould, A.R., Kattenbelt, J.A., Selleck, P., Hansson, E., Della-Porta, A.J. and Westbury, H.A. (2001). Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998-2000. *Virus Research* **77**: 7751-7760.

Hooper, P.T., Hansson, E., Young, J.G., Russell, G.M. and Della-Porta. A.J. (1999a). Lesions in the upper respiratory tract in chickens experimentally infected with Newcastle disease viruses isolated in Australia. *Aust. Vet. J.* **77**: 50-51.

Hooper, P.T., Russell, G.M., Morrow, C.J. and Segal, Y. (1999b). Lentogenic Newcastle disease virus and respiratory disease in Australian broiler chickens. *Aust. Vet. J.* **77**: 53-54.

Hooper, P.T., Gould, A.R., Hyatt, A.D., Braun, M., Kattenbelt, J.A., Hengstberger ,S.G. and Westbury, H.A. (2000). Identification and molecular characterisation of Hendra virus in a horse in Queensland. *Aust. Vet. J.* **78**: 281-282.

Lamb, R.A. and Kolakofsky, D. (1996). Paramyxoviridae: The viruses and their replication. In; Fields Virology, Third edition ed by Fields, B.N., Knipe, D.M., Howeley, P.M. *et al*., Raven Publishers, Philadelphia. pp 1177- 1204.

Nagai, Y. and Klenk, H.D. (1977). Activation of precursors to both glycoproteins of Newcastle disease virus by proteolytic cleavage. *Virology* **77**: 125-134.

de Leeuw, O. and Peeters, B. (1999). Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. *J. Gen. Vir.* **80**: 131-136.

Nagai, Y., Ogura, H. and Klenk, H.D. (1976). Studies on the assembly of the envelope of Newcastle disease virus. *Virology* **69**: 523-538.

Nagai, Y., Klenk, H.D. and Rott, R. (1976). Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* **72**: 494-508.

Ogasawara, T., Gotoh, B. Suzuki, H., Asaka, J., Shimokata, K., Rott, R. and Nagai, Y. (1992). Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. *EMBO J.* **11**: 467-472.

Page, R.D.M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357-358.

Peeters, B.P.H., de Leeuw, O.S., Koch, G. and Gielkens, A.L.J. (1999). Resue of Newcastle disease virus from cloned cDNA: Evidence that cleavability of the fusion protein is a major determinant for virulence. *J. Virol.* **73**: 5001-5009.

Römer-Oberdörfer, A., Mundt, E., Mebatsion, T., Buchholz, U.J. and Mettenleiter, T.C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. *J. Gen. Virol.* **80**: 2987-2995.

Sakaguchi, T., Toyoda, T., Gotoh, B., Inocencio, N.M., Kuma, K., Miyata, T. and Nagai, Y. (1989). Newcastle disease virus evolution: I. Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. *Virology* **169**: 260-272

Sato, H., Hattori, S., Ishida, N., Imamura, Y. and Kawakita, M. (1987). Nucleotide sequence of the hemagglutinin-neuraminidase gene of Newcastle disease virus avirulent strain D26: Evidence for a longer coding region with a carboxyl terminal extension as compared to virulent strains. *Virus Research* **8**: 217-232.

Simmons, G.C. (1967). The isolation of Newcastle disease virus in Queensland. *Aust. Vet. J.* **43**: 29-30.

Toyoda, T., Sakaguchi, T., Hirota, H., Gotoh, B., Kuma, K., Miyata, T. and Nagai, Y. (1989). Newcastle disease virus evolution: II. Lack of gene recombination in generating virulent and avirulent strains. *Virology* **16**: 273-282