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The molecular basis of the pathogenicity of Newcastle disease virus in chickens

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ABSTRACT

Newcastle disease (ND) is a highly pathogenic disease of poultry and is caused by virulent strains of Newcastle disease virus (NDV). From 1998-2002 there were outbreaks of ND in Australia which resulted in significant disruptions to the poultry industry. In some of these outbreaks however, the clinical signs observed in the infected birds did not appear to correlate with the World Organisation for Animal Health's definition of a virulent virus, which is based on the molecular sequence at the fusion protein cleavage site. In one particular outbreak at Meredith, Victoria, in 2002, a virulent virus was isolated, despite only a minimal increase in mortalities on the property. Therefore, this thesis has attempted to determine whether, in addition to the fusion protein cleavage site, there are other molecular determinants of pathogenicity for NDV.

The pathogenicity of the Meredith/02 virus was first characterised by experimental infection of specific pathogen free (SPF) chickens. The Meredith/02 virus was compared with an avirulent virus (Peats Ridge/98) and two other virulent viruses (Herts 33/56 and Texas GB) using clinical evaluation, histopathology, immunohistochemistry and molecular techniques. The Meredith/02 virus showed minimal clinical signs in a small number of birds and no mortalities. The birds infected with Herts 33/56 and Texas GB were all euthanased at day 2 post inoculation and day 5 post inoculation respectively. The minimal pathogenicity of the Meredith/02 virus was associated with decreased virus replication and antigen distribution in a number of tissues when compared with the Herts 33/56 and Texas GB viruses.

Further characterisation of the Meredith/02 virus showed that it contained a virulent fusion protein cleavage site motif of ^{112}RRQRRF^{117}, which is exactly the same as the cleavage site of Herts 33/56. The mean death time in eggs classified the virus as a mesogenic NDV. Sequence analysis showed a number of amino acid differences throughout the genomes of the four viruses studied, however none of these differences were in key areas such as glycosylation sites. The Meredith/02 virus was also shown to replicate well in embryonated eggs, throughout the chorioallantoic membrane and internal organs of the embryo, including the lung, liver and kidneys. This is consistent with other virulent NDVs.

The V protein of the Meredith/02 virus was investigated for its role in potential attenuation of the virus via modulation of the host innate immune response. However there was no difference found in the ability of the Meredith/02 V protein to antagonise type I interferon *in-vitro* when compared with the Herts 33/56 virus.

In an attempt to analyse the viral replication complex, to identify a specific protein that may be involved with the minimal pathogenicity of the Meredith/02 virus, the transcription gradient of the virus was characterized. It was found that the Meredith/02 virus has an increased transcription gradient when compared with the Herts 33/56 virus. The gradient of the Meredith/02 virus was particularly steep at the N-P junction, with particularly low levels of the P gene transcribed at 24 hours. However, gene start and end sequences at this location did not vary between the two viruses, thereby indicating that the N and P proteins are less likely to be associated with the steepened gradient. Instead, this suggests a possible role for the large polymerase protein in decreasing transcription.

Whilst this research has not yet identified specific molecular sequences responsible for the minimal pathogenicity of the Meredith/02 virus despite its virulent fusion protein cleavage site, it has focused the investigation on components of the viral replication complex. Therefore directions for further research include investigating the role of the replication complex, in particular, the large polymerase (L) gene in the pathogenicity of Australian NDVs. This could also incorporate further work on the individual proteins via the use of minigenome assays, or by utilising reverse genetics and full-length virus clones. Additional transcriptional profiles of other mesogenic viruses could also be analysed. It would also be interesting to compare the pathogenicity of the Meredith/02 virus with other viruses from the 1998-2002 Australian outbreaks in an experimental setting.

The outcomes from this work have provided greater insight into an Australian NDV which until now has not been well characterised. This research is also relevant to the broader group of mesogenic NDVs which are not easily classified according to their fusion protein cleavage sites.

DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Contributor	Statement of contribution
J. Bergfeld	Conceived experiments (20%) Designed experiments (25%) Wrote the paper (100%)
J. Meers	Conceived experiments (20%) Designed experiments (25%) Edited the paper (70%)
J. Bingham	Conceived experiments (20%) Designed experiments (25%) Edited the paper (20%)
J. Harper	Technical assistance with histology
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G. Marsh	Technical advice regarding PCR
M. Tachedjian	Whole genome sequence assembly
D. Middleton	Conceived experiments (40%) Edited the paper (10%)

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No contributions by others.

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None.

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KEYWORDS

Newcastle disease virus, Australia, poultry, chickens, pathogenicity, virulence, molecular

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

aa	amino acid
BLAST	Basic local alignment search tool
bp	base pair
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
CEF	Chicken embryo fibroblast
CNS	Central nervous system
CPE	Cytopathic effect
C_T	Cycle threshold
DMEM	Dulbecco modified eagle medium
dpc	days post challenge
GFP	Green fluorescent protein
HI	Haemagglutination inhibition
HR	Heptad repeat
ICPI	Intracerebral pathogenicity index
IFN	Interferon
IHC	Immunohistochemistry
ISG	Interferon stimulated gene
JAK	Janus kinase
LEADDR	Laboratories for emergency animal disease diagnosis and response
LB	Luria-Bertani broth
MDT	Mean death time
Mx	Myxovirus resistance protein
ND	Newcastle disease
NDV	Newcastle disease virus
NDV F	Newcastle disease fusion gene
NDV HN	Newcastle disease haemagglutinin-neuraminidase gene
NDV L	Newcastle disease large polymerase gene
NDV M	Newcastle disease matrix gene
NDV N	Newcastle disease nucleoprotein gene
NDV P	Newcastle disease phosphoprotein gene
ng	nanogram

NSW	New South Wales
nt	nucleotide
OIE	World Organisation for Animal Health
qPCR	Quantitative polymerase chain reaction
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
Poly I:C	Polyionsinic:polycytidylic acid
RPM	Rotations per minute
RNAP	RNA-dependent RNA polymerase
rRNA	Ribosomal ribonucleic acid
SPF	Specific pathogen free
SOP	Standard operating procedure
STAT	Signal transducer and activation of transcription
TLR	Toll like receptor
$\Delta\Delta C_T$	Delta delta C_T
µl	microlitre

CHAPTER 1
INTRODUCTION

Viruses were first discovered as a cause of disease during the late 1800s with the discovery of the tobacco mosaic virus.[1] Ever since this time, researchers have investigated the properties of viruses that make them pathogenic in a particular host. The pathogenicity of a virus can be described in numerous ways, including the ability to replicate, transmit or to cause disease. In understanding the factors that enable these viruses to exert their damaging effects, it is hoped that diagnostic techniques, therapeutics and preventative measures such as vaccines may be developed to counteract the viral machinery. Molecular analysis of the viral genome has provided immense insights into the molecular basis for pathogenicity for a number of viruses such as avian influenza virus and Newcastle disease virus (NDV) .[2, 3] The use of cloning techniques has allowed for specific sequences and proteins to be examined in isolation and manipulated in an experimental setting. This has also led to the development of diagnostic tests to target specific pathogenic sequences which are then able to inform risk assessments and control measures for the pathogen in question.

This thesis investigates the molecular basis for pathogenicity of NDV in poultry. NDV is a paramyxovirus which is able to infect a wide range of avian species and can be particularly pathogenic in chickens. Similar to avian influenza virus, it has a range of pathogenicity which varies with the virus strain and the host species. Newcastle disease (ND) has significant impacts throughout the much of the world in areas of Central and South America, Asia, the Middle East and Africa, where it is endemic.[4] It is also a significant biosecurity risk in ND free countries where sporadic outbreaks can have great impacts on trade. Australia is currently free of virulent ND, however outbreaks have occurred in the past, with the most recent outbreaks occurring between1998-2002.

The significance of ND is reflected in its status as a notifiable disease by the World Animal Health organization.[5] Only virulent strains of NDV are able to cause ND and are therefore notifiable. As such, the definition of a virulent NDV strain is particularly important and is currently based upon a known determinant of pathogenicity, the fusion protein cleavage

site.[6] In particular, the molecular sequence of multiple basic amino acids at the cleavage site, along with a phenylalanine at position 117, has been correlated with pathogenicity.[7, 8]

In order to further investigate the molecular basis for NDV pathogenicity, this work has focused on an outbreak of ND in Australia in 2002. During this outbreak a virus was isolated from a layer property in Meredith, Victoria. Molecular sequencing of the fusion protein of this virus showed that it had a cleavage site motif that was classified as virulent, however the clinical signs observed in the field did not appear to correlate well with this classification. The clinical signs were mild and mortalities were lower than expected. Therefore, it was hypothesized that there may be other molecular determinants of pathogenicity, other than this cleavage site motif. The overall aim of this research is to identify these other determinants of pathogenicity.

Chapter 2 of this thesis reviews the current literature surrounding ND in Australia, including a summary of research into molecular pathogenicity determinants for NDV. The methodology used throughout this work is presented in detail in Chapter 3, however methods specific to certain sections will also be included in the relevant chapter.

The clinical signs that were observed in the poultry involved with the outbreak at Meredith were fairly mild, with the most significant observations being a drop in egg production and only a minimal increase in mortalities over the outbreak period.[9] In order to further investigate the phenotype associated with the virus, it was necessary to determine whether these observed clinical features were reproducible. As such, chickens were inoculated with the Meredith/02 virus experimentally and various measures of pathogenicity were assessed. This work is presented in Chapter 4.

After investigating the pathogenic features of the Meredith/02 virus, it was further characterized by standard virological techniques in Chapter 5. These studies were conducted in order to develop baseline parameters that would allow for comparisons to be made with other ND viruses. These measures included virus isolation, mean death time in eggs, histopathology of infected chicken embryos and whole genome sequencing.

Based on results from previous studies that identified the NDV V protein as an antagonist of the chicken innate immune system, Chapter 6 investigated the V protein of the

Meredith/02 and its role in pathogenicity.[10, 11] The Meredith virus was compared with the velogenic Herts 33/56 virus for its ability to induce interferon expression and to antagonize interferon-α, interferon-β and Mx *in-vitro*.

In Chapter seven, the transcriptional profile of the Meredith/02 virus was compared with the Herts 33/56 virus. This work was undertaken to attempt to identify particular NDV genes which may be associated with the attenuated phenotype of the Meredith/02 virus and to follow up on work that has suggested that the viral replication complex may be associated with pathogenicity.[12, 13]

Chapter 8 concludes this thesis with a discussion of the research undertaken and recommendations for further investigations.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Newcastle disease (ND) is an extremely important viral disease of poultry and wild birds worldwide. It is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) with APMV-1 being synonymous with Newcastle disease virus (NDV).

The OIE defines ND as an 'infection of poultry caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater,
   or
b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and a phenylalanine at residue 117.'[6]

The disease rivals avian influenza in its impacts on trade, animal welfare and biodiversity and is therefore an OIE notifiable disease.[5] In the developing world, poultry production is a significant contributor to poverty alleviation and therefore ND outbreaks can also have vast socioeconomic consequences.[14]

2.2 History

2.2.1 Worldwide occurrence

ND is so named because it was first recognised as a cause of disease in poultry in Newcastle upon Tyne in England by Doyle in 1926.[15] Doyle was the first person to investigate the aetiological agent of the disease via laboratory experiments and noted that it was a filterable virus that was unrelated to avian influenza virus. However, the first known reports of the disease were from outbreaks in Jakarta, Indonesia in the previous year, in which very high mortalities were seen in affected poultry.[16, 17] The exact origin of

the virus is still unknown, however given that the clinical signs of avian influenza and ND are similar, it is possible that the virus had been circulating in poultry over an extended period of time without the recognition that it was a distinct entity. In fact, there were reports of disease in Europe and Scotland that resemble ND before 1926.[18, 19]

The spread of ND throughout the world during the 20th century occurred via a number of panzootics, although the waves of disease were not always distinct and the epidemiology of the disease varied significantly between affected countries. The history of ND throughout the world has been extensively reviewed in a number of publications by Alexander but in particular, in his publication of 2001.[20]

The first panzootic occurred during the 1920s and 1930s with viruses detected throughout the world that were very similar to the strain isolated by Doyle. However, whilst these viruses were almost identical to the original NDV, the range of clinical signs that they produced in poultry were quite variable.[21] The highly pathogenic forms of ND initially spread throughout Indonesia, England and India. In India the disease was termed 'Ranikhet disease' after the town in which it was first seen in 1927, a name which is still in use today.[22, 23] It has also previously been known as 'Fowl Pest', although confusingly this term is also used to refer to avian influenza virus infection.[24] It is debatable as to how long this wave of disease continued, although researchers believe that it took more than two decades to establish in most countries.[19] During this time, two reference strains of ND were isolated; Texas GB and Herts 33.[25] These viruses are now used as challenge strains in vaccine production.

Whilst the original reports of NDV were typically associated with the highly pathogenic, viscerotropic form of the disease, a slightly milder strain of the virus was later detected and studied, after isolation in California from chickens with neurological and respiratory signs.[26] The disease was termed pneumoencephalitis and it was shown by Beach that the virus could increase in virulence for chickens with repeated passage in chickens and eggs. This form is consistent with neurotropic velogenic NDV.[21]

The second panzootic of NDV is thought to have started during the 1960s and took a considerably shorter time to spread throughout the world compared with the previous wave of disease. It is estimated that it only took four years for the disease to be detected in

most countries.[20] The greater speed of spread is most likely associated with increased commercialisation of poultry and poultry feed, along with faster transportation speeds.[19] In particular, the movement of caged birds is known to have been key in the introduction of disease into the USA, in which psittacine and mynah birds were linked to outbreaks of disease.[27] In 1970, high mortalities of birds in a pet shop in New York City were reported and ongoing outbreaks of disease in captive birds were also seen over the next few years from 1970-1972. Disease outbreaks in poultry were also investigated during this time and were often associated with the importation of game birds or other poultry, in some cases from Mexico or Puerto Rico.[27] After this, more stringent quarantine restrictions were enforced on the importation of birds to the USA.

During the 1970s there were a small number of reports of another virulent NDV spreading throughout the world, however the next true third panzootic occurred during the 1980s and was associated with pigeons.[19] A virus isolate obtained from a pigeon in Iraq in 1978 was identified as an avian paramyxovirus-1 with similar characteristics to NDV.[28] The virus is now known as pigeon paramyxovirus (PPMV-1). The viruses found in the Middle East then spread to Europe in the early 1980s.[29] After this, there were detections of the pigeon virus in poultry, often as a result of contaminated feed, as seen in Great Britain in 1984.[30]

The fourth panzootic is ongoing and started in the mid-1980s in Southeast Asia. The viruses responsible for this panzootic are from genotype VII.[4, 31] Currently a potential fifth panzootic has been described with the circulation of viruses from sub-genotypes VIIh and VIIi, likely originating from Indonesia in 2009.[32, 33]

Whilst ND is still prevalent worldwide, virulent forms are controlled in most developed countries by the use of strict biosecurity protocols and vaccination. However, even with access to vaccination, outbreaks still occur.[34, 35] In addition, APMV-1 strains continually circulate in wild birds, at times causing mass mortalities particularly in double-crested cormorants and posing ongoing risks to commercial poultry.[21]

2.2.2 Newcastle disease in Australia

ND has been seen in Australia on only a few occasions since it was first reported in the 1920s. However, avirulent NDV is known to circulate in all parts of Australia with ongoing

detections in wild birds and poultry.

1930s: Virulent NDV in Victoria

ND was first detected in Australia in 1930 in Inverloch, Victoria. The disease subsequently spread throughout Melbourne before it was brought under control. A second outbreak occurred in 1932, also in Victoria and was eradicated in 1932.[36, 37] The virus isolated from the 1932 outbreak is the oldest isolate of NDV from Australia and is known as the Albiston-Gorrie strain.

1960s: Avirulent endemic NDV strains

In 1963, chickens exported from Australia to Malaysia were reported to be infected with ND, however serosurveys conducted in response to this claim did not find any evidence of the virus in the Australian poultry flock [37, 38]. The next detection of ND in Australia was not made until 1966, when a lentogenic strain termed 'V4' was found in broilers in Queensland as part of a mixed infection with other viral and parasitic agents [39]. Experimentally, the virus was shown to have an intracerebral pathogenicity index (ICPI) of 0.1, consistent with an avirulent virus and inoculation of embryonated eggs did not produce embyro deaths.[40] However subsequent testing of the V4 strain showed higher ICPIs of 0.91 and 1.02.[41] The V4 virus was then found to be widespread throughout Australia, although it was never shown to cause clinical disease on its own.[42, 43] As a result of this lack of pathogenicity, no attempt was made to eradicate the virus and it was then investigated for use as a vaccine due to its protective effects against challenge with the virulent Albiston-Gorrie NDV strain.[44, 45] Further avirulent viruses were also isolated during the 1960s and 1970s.[46, 47] Genetically, these Australian viruses were distinct from exotic ND viruses and it is not known where they originated from, although spillover from wild birds was suggested when serological surveys in wild waterfowl demonstrated the presence of NDV antibodies.[48] However, testing of wild birds thought likely to introduce NDV into Australia in far north Queensland did not detect any ND virus or antibodies. This testing involved both serology and virus isolation from 130 species, including pittas, pigeons, herons, gulls and terns.[49]

In comparison with other virulent NDV strains, these Australian lentogenic strains were found to have extended open reading frames of the haemagglutinin-neuraminidase (HN) gene of 45 amino acids.[50] It was thought that this HN extension may be associated with the low pathogenicity of the V4 strain.[41]

1978: Avirulent NDV in an imported cockatoo

The next isolation of a genetically distinct ND virus in Australia was in 1978, when an illegally imported cockatoo from Indonesia was found to be infected.[51] This virus was a lentogenic strain and did not cause severe disease, unlike the strains that caused outbreaks in psittacines in the United States in the 1970s. It is now commonly referred to as the Eaves-Grimes strain.

1980s-1990s: Evolution of avirulent ND viruses

Throughout the 1980s and 1990s, detections of NDV from chickens continued and were all determined to be avirulent viruses. Some of these viruses had been isolated from birds with respiratory signs, however infectious bronchitis virus was usually deemed to the be the aetiological agent of disease in these cases.[50]

1998-2002: Virulent NDV outbreaks in New South Wales and Victoria

During August 1998, an isolate of NDV was detected in a flock of poultry from Peat's Ridge in NSW. This virus was isolated from birds that were showing signs of a syndrome termed 'late respiratory disease', which was thought to be caused by a combination of agents such as infectious bronchitis virus, *Mycoplasma* sp., *Escherichia coli* and possibly NDV.[52] This virus was considered avirulent and had an F protein cleavage site motif of RRQGRL, consistent with avirulent strains. It also had an HN extension of 9 amino acids, indicating a significant evolutionary change from previous Australian isolates.[53]

It was not long after the investigation of 'late respiratory disease' at Peat's Ridge, that the first outbreak of virulent NDV since 1932 was confirmed in Australia in September 1998.[54-56] Sequencing of the F gene cleavage site of this virus showed a virulent motif of RRQRRF which was only two nucleotides different from the avirulent sequence from Peats Ridge.[53] The cleavage site of ancestor ND viruses is typically well conserved and the fusion protein as a whole is under negative selection pressure, indicating that unusual circumstances may have been involved in the generation of this virulent virus.[57]

As a result of the detection of this virus, an eradication campaign was instigated. Outbreaks then occurred throughout 1998 until 2002, in New South Wales and Victoria and caused great disruption to the Australian poultry industry along with the

implementation of a mass vaccination program. A summary of these outbreaks during this period is shown in Table 2.1 with a map of the location of outbreaks in Figure 2.1.

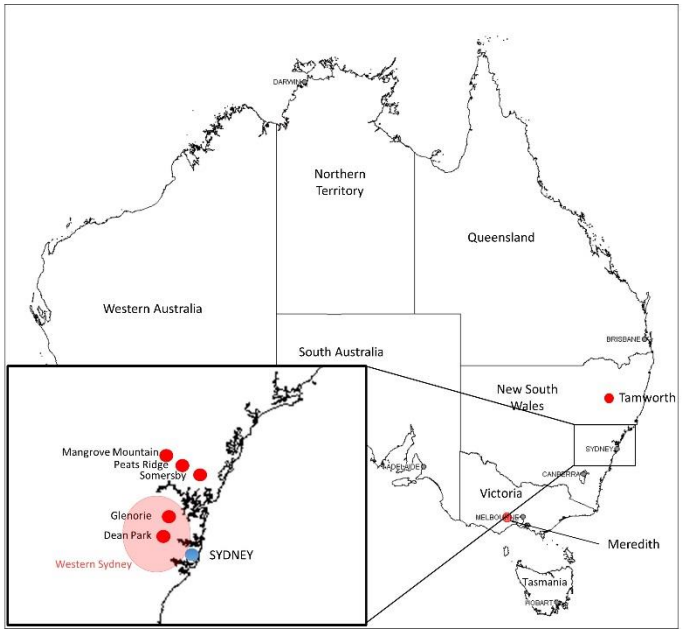


Figure 2.1 Map of Australian ND outbreak locations (1998-2002)

The initial outbreaks in 1998 occurred in western Sydney at Dean Park and Glenorie and again Rylstone, all in New South Wales.[55, 56] Whilst the viruses involved were designated as virulent with ICPIs between 1.64 and 1.9, it was noted that they had reduced transmissibility compared with other known NDV isolates.[55] However, in most cases the clinical signs seen during these outbreaks were consistent with velogenic neurotropic NDV, including torticollis and flaccid paralysis.[58] The outbreaks were controlled by stamping out and the implementation of restricted movement zones as per government protocol. In addition, sentinel chickens were placed on farms to detect the presence of residual virus.[56]

However, in April 1999 NDV was once again detected in the Mangrove Mountain region of NSW, an area with a high density of poultry production. This detection was the start of the greatest outbreak of NDV in Australia to date.[56] Control of the Mangrove Mountain outbreak involved the culling of approximately two million birds at a cost of roughly $26 million AUD.[59, 60] Vaccination was then instituted in December 1999 using the live V4 vaccine.[60] Subsequent Australia-wide surveillance for NDV in 2000 showed that there was

a prevalence of 39.8% seropositive farms, although no virulent viruses were detected at this time.[61, 62]

Two years after the disease events in NSW, Victoria also experienced an isolated outbreak. In May 2002, a layer farm at Meredith was found to be infected with virulent NDV. This outbreak differed from the previous outbreaks in that the clinical signs were not as severe as previously seen. The major sign involved in this case was a drop in egg production of approximately 40% with only a very slight increase in mortality in one shed; from 0.4 to 0.8% per month.[9] However, the Meredith isolate as per the other 1998-2002 Australian isolates contained a virulent F protein cleavage site (RRQRRF) and hence was reportable to the OIE. As a result, the Meredith flock was culled to eliminate the disease.[9]

Finally, the last outbreaks of virulent NDV in Australia were detected in October and November 2002 in Horsley Park near Sydney. After depopulation of these properties, widespread surveillance was undertaken to ensure that there were no other virulent viruses present within the Australian poultry flock. Then in June 2003, Australia was officially recognised by the OIE as being free from virulent ND and no further outbreaks have occurred since that date.[63]

Date	Location	Species	Clinical signs	Reference
September 1998	Blacktown = Dean Park (WS)	Mixed poultry: caged layers, free-range layers, broilers, pullets, ostriches, geese, one duck and feral pigeons	30% case mortality rate	[56, 58]
September 1998	Glenorie (WS)	Caged and free-range layers	Increased mortalities and neurological disease	[58] (unpublished data)
September 1998	Rylstone	Free-range broilers	N/A	[58]
April 1999	Mangrove Mountain (near Gosford)	15 layer and broiler farms	Increased mortalities and neurological disease	[56]
August 1999	Schofields (WS)	N/A	N/A	[56]
December 1999 – February 2000	Orchard Hills (WS)	Layer	Minimal disease, mild ataxia	[56, 64] (unpublished data)
	Llandilo (WS)	Layer	Minimal disease, poor shells	[56, 64] (unpublished data)
	Marsden Park (WS)	N/A	Mild increase in mortalities and neurological signs	(unpublished data)
	Rossmore (WS)	N/A	Occasional birds with neurological signs	(unpublished data)
February 2000	Moonbi (Tamworth)	Layer	Increase mortality with nervous signs	[56] (unpublished data)
May 2002	Meredith (Victoria)	Layer	Egg drop	[65]
October – November 2002	Horsley Park (WS)	Layer	N/A	[66, 67]

Table 2.1 Outbreaks of ND in Australian from 1998-2002 (WS: Western Sydney; N/A: not available)

During these outbreaks significant questions were raised regarding the origin of the viruses. It was therefore necessary to determine whether the initial outbreak had occurred due to an exotic isolate or due to a mutation of the endemic avirulent strains. Molecular sequencing of these strains was undertaken in the first few days of the outbreak and showed that they were unique to Australia and had most likely arisen from the 1998 Peat's Ridge isolate, now termed the 'progenitor' virus.[53] In fact, the progenitor strain was detected on the vast majority of affected farms in conjunction with a virulent virus. Sequencing of the HN gene of the progenitor virus and the virulent outbreak strains showed that they all had a 9 amino acid extension that was similar to two of the previous

avirulent Australia viruses (NSW 12/86 and Qld 1/87).[53] This extension had not been seen elsewhere in the world and as such it was presumed that the virulent viruses had arisen from endemic Australian strains.

2.3 Aetiology

NDV is a member of the family *Paramxyoviridae*, subfamily *Paramyxovirinae* and genus *Avulavirus*. It is a single-strand, negative sense RNA virus, as per the other members of the family. Along with NDV (APMV-1) and PPMV-1 (APMV-1, genotype VI), the genus *Avulavirus* contains the avian paramyxoviruses serotypes 2-12 and a putative APMV-13 serotype.[68, 69] ND is defined by the OIE as infection of poultry with virulent forms of APMV-1.[6]

2.3.1 Structure

The genome of NDV is approximately 15 kb long with most current isolates having a genome length of 15,192 nt or 15,198 nt. ND viruses isolated prior to 1960 predominantly had genomes of 15,186 nucleotides.[70] The Australian ND viruses isolated from the 1998-2002 outbreaks also have genome lengths of 15,186 nt.[71] The viral genome contains six genes, from 3' to 5'; nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), haemagluttinin-neuraminidase (HN) and the large RNA polymerase (L). The six genes encode seven proteins with transcriptional editing of the P gene producing the V protein (Figure 2.2).

As per the other members of the paramyxovirus family, NDV is an enveloped virus and contains both fusion (F) and haemagglutinin-neuraminadase (HN) glycoproteins proteins on its surface. The virions are spherical in shape and the envelope is produced by budding from the host cell.[72] Within the virions, the proteins associated with the helical nucleocapsid are the N, P and L proteins. The M protein is a structural protein, which is found on the inner surface of the lipid envelope.

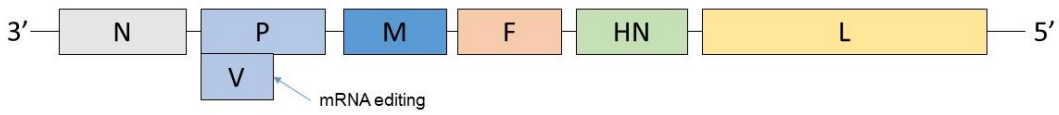


Figure 2.2 NDV RNA genome showing the V protein which is produced via RNA editing of the P gene

2.3.2 Virus entry

The envelope proteins are responsible for viral entry into a target cell. The HN protein mediates cell attachment, whereas the F protein is necessary for cell fusion. The HN protein requires sialic acid residues on the surface of the host cell in order to bind. It then

also plays a role in viral release from the cell via its neuraminidase activity which removes sialic acid receptors.[73] The fusion protein is a key component of the pathogenesis and virulence of the virus, as the ability of the virus to enter a cell is dependent upon the cleavage of the inactive F0 protein by cellular proteases. This is discussed further in the section on pathogenicity determinants (Chapter 2.8.3). After fusion of the virus envelope with the host cell membrane, the viral nucleocapsid is released into the cytoplasm.

2.3.3 Replication

After entry into the cell, transcription of the negative sense viral genome occurs in the cytoplasm. The RNA-dependent RNA polymerase (RdRP) transcribes the leader RNA and each of the viral genes into individual 5' capped and 3' polyadenylated mRNAs, along a transcription gradient.[74] This leads to larger numbers of mRNA transcripts being produced from genes closest to the promoter region when compared with genes closer to the 5' end. The individual mRNAs are then translated into viral proteins.

After sufficient numbers of viral proteins are produced, transcription stops and replication begins. Replication produces a full-length antigenome of the negative sense RNA, in association with the N protein.[75] Each of the N subunits is associated with 6 nucleotides of genomic RNA and hence adheres to the 'rule-of-six' of most paramyxoviruses.[76] This feature explains why the genome sizes of all NDVs are always a multiple of six.

2.3.4 Assembly and Release

Nucleocapsids assemble in the cytoplasm of the host cell with initial attachment of the N protein to the RNA to form a helix followed by integration of the P and L proteins. The nucleocapsids are then transported to the plasma membrane and are connected to the F and HN surface glycoproteins via the M protein. The viral envelope is formed during the process of budding from the host cell.[75]

2.4 Classification

Whilst NDVs are of a single serotype, they can be classified genetically in a number of ways. They have traditionally been classified on the basis of the full length F gene sequence and have been described either in terms of lineages and sub-lineages, or classes and genotypes.[77-80] The use of multiple classification systems has led to

confusion and so efforts have been made to determine the most appropriate universal system. Such a system has been developed based on F gene phylogeny and evolutionary distances.[81] The fusion gene is usually chosen for comparative analysis because it is more likely to show genetic variation than other internal nucleocapsid genes.[77] For the purposes of this thesis, the genotype classification system will be used as it is currently the most widely utilized.

Using this classification system, two clades of NDV are recognised; class I and class II. Class I viruses have a genome of 15,198 nt, whereas class II viruses have a genomes of 15,186 nt ("historic" isolates) and 15,192 nt (isolated after 1960).[78] The majority of the class I viruses have been isolated from wild birds (predominantly waterfowl and shorebirds) and are avirulent, whereas the class II viruses contain isolates from both wild birds and poultry and contain both virulent and avirulent strains.[70] These two classes can then be further classified into genotypes. Class I viruses contain a single genotype, whereas Class II viruses contain 18 genotypes.[82]

2.5 Epidemiology

2.5.1 Hosts

NDV has the ability to infect a wide variety of avian species, however the pathogenicity of the virus amongst species is variable. Poultry are most susceptible to ND, with high mortalities seen in layer and broiler farms. Other commercial species such as turkeys and ducks are also known to be susceptible to ND but clinical signs are typically less severe than those seen in chickens.[83] In addition, viruses with virulent fusion protein cleavage sites have been detected in commercial ducks with no apparent clinical signs seen in the infected birds, thus posing a potential risk to other poultry species.[84]

Whilst waterfowl are thought to be reservoirs of avirulent ND viruses, the epidemiology of these viruses in wild birds is unclear.[85] However, the potential for these viruses to become virulent was seen experimentally when a lentogenic NDV isolated from a migratory goose was passaged in chickens, resulting in a virulent fusion protein cleavage site but only mild clinical signs in poultry.[86]

Disease associated with NDV infection has also been seen in significant outbreaks in wild birds such as cormorants, pigeons, pelicans, gulls and peacocks.[87-90] In 1990, an epizootic of ND occurred in double-crested cormorants (*Phalacrocorax auritus*), white pelicans (*Pelecanus erthrorhynchos*) and gulls (*Larus* spp.) in Canada.[91] Similar outbreaks in cormorants were also seen in North America. Subsequently, viruses very similar to these wild bird isolates were found in turkeys in North Dakota and it is postulated that the turkeys were infected by the wild birds.[92] Migratory birds have also been implicated in the infection of chickens and turkeys in Great Britain in 1997.[93] In addition, wild pigeons and feed contaminated with pigeon faeces were thought to be responsible for ND outbreaks in poultry the UK.[94]

NDV is also recognised as a zoonotic agent. The predominant clinical sign shown by those infected is conjunctivitis, however there have also been occasional reports of ND causing flu-like symptoms.[21, 95] The majority of people reported to be infected with NDV are those with close contact with poultry (e.g. abattoir workers) or laboratory staff. As yet there have been no reports of human to human spread.

2.5.2 Transmission

The majority of ND outbreaks occur as a result of disease spread from infected poultry.[83] Introduction of disease can occur from a number of sources including trade in poultry and poultry products and via the smuggling of live birds or eggs. Once the disease has been established in a flock, spread of the virus is typically by movement of birds, via fomites (feed, equipment) and potentially by windborne dispersal.[96]

During an outbreak, bird to bird transmission is usually via the respiratory route with inhalation of droplets, or via the faecal-oral route.[96]

2.6 Clinical Signs

The clinical signs of ND may vary with the pathotype of the virus. Four main pathotypes were described in the 1970s and the terminology is still in use today. The pathotypes include velogenic (highly virulent), mesogenic (moderately virulent), lentogenic (low virulence) and avirulent forms which have been derived from the mean death time (MDT)

in eggs. The velogenic viruses can then also be further divided into velogenic viscerotropic and velogenic neurotropic forms based on pathological features.[25] In general, velogenic viruses are associated with high mortalities with viscerotropic viruses causing severe depression and diarrhoea. Neurotropic viruses also cause neurological signs such as ataxia, head tremors and paresis, along with respiratory distress. Mesogenic viruses usually present with respiratory disease and may also cause mortalities in young birds. Lentogenic viruses induce minimal clinical signs, however when present they are usually respiratory in nature.[6] Avirulent viruses typically do not induce clinical signs at all. There is however overlap between the pathotypes and the age, immune status and the presence of concurrent diseases should be taken into consideration when interpreting the clinical signs.

2.7 Pathology

The gross and histological features of ND vary with the pathotype of virus. Velogenic viruses have a strong tropism for lymphoid tissue and the central nervous system.[97] Infection with viscerotropic, velogenic pathotypes typically produces haemorrhagic lesions within the intestinal tract which can be attributed to necrosis of lymphoid tissue within these regions. In particular, fibrinonecrotic lesions are commonly seen in the caecal tonsils and the small intestine.[98] Spleens are often enlarged and mottled and the proventriculus may contain haemorrhagic foci, again usually centred on lymphoid aggregates. These gross findings correspond histologically to necrosis of lymphoid tissue with replacement haemorrhage and fibrin. Immunohistochemical staining and in situ hybridization has shown that the lymphoid tropism observed is usually associated with replication of virus in macrophages.[98, 99] Initially, replication occurs within lymphocytes and macrophages in the eyelid conjunctiva.[100] This progresses to the detection of positive staining in mononuclear cells throughout multiple organs, along with positive staining in epithelial cells of the respiratory and gastrointestinal tracts.

Infection with neurotropic velogenic strains show minimal lesions grossly, however respiratory disease with haemorrhagic tracheitis and increased exudate within the bronchioles and trachea may be present. Histologically, lesions are consistently seen throughout the central nervous system. They consist of non-suppurative inflammation

predominantly perivascularly, along with gliosis and neuronal degeneration. The distribution of lesions is important for sample collection, as lesions are most frequently seen throughout the caudal CNS (spinal cord, medulla, brain stem), with minimal lesions present in the cerebrum.[21] The ability of the various pathotypes of NDV to cause lesions in the brain may be associated with differences in peripheral replication and the ability to invade neural tissue.[101, 102] Whilst both mesogenic and velogenic viruses have the ability to invade neural tissue, the rate of replication of mesogenic viruses is comparatively slower. Lentogenic viruses do not appear to have the ability to replicate in neural cells at all. In some cases, viscerotropic pathotypes may cause death of birds before neurological lesions have time to develop.[102]

Experimentally, mesogenic viruses show limited gross pathological changes apart from splenic enlargement and increased air sac opacity.[98] This corresponds to lymphoid hyperplasia within the spleen and lymphoid follicle formation within the air sacs. In addition, mononuclear myocardial inflammation may also be present.

Minimal gross findings are seen with lentogenic strains, although histologically, there may be airsaculitis or tracheitis.

Whilst ND is typically non-pathogenic in wild birds, in outbreaks of disease in double-crested cormorants in the United States and Canada, lesions were typically of a neurotropic pathotype. Minimal gross lesions were seen, however histologically, there was evidence of mononuclear perivascular cuffing in the brain and spinal cord, along with gliosis and white matter vacuolation.[91]

In Australia, investigations into ‘late respiratory syndrome’ of broilers, identified the presence of lentogenic NDV in cases where tracheitis had been diagnosed histologically in conjunction with *E. coli* infection.[52] Further experimental work using Australian lentogenic isolates of NDV showed that they were able to cause conjunctivitis with varying degrees of tracheitis. This inflammation was represented histologically by lymphocyte infiltration and antigen was found present within the lesions.[103]

The pathological features of disease seen during the 1998-2002 outbreaks of ND in Australia are poorly described in the literature. The AUSVETPLAN manual notes that

there were few gross pathological changes seen during these outbreaks, however perivascular lymphocytic cuffing in the brainstem was seen microscopically.[83] One of the Australian strains isolated during 1998 from Glenorie has been used in two experimental trials in the United States and the virus was described as velogenic viscerotropic.[101, 104] All birds infected with this virus showed slight depression with mild neurological signs, along with one very sick bird but all recovered by the end of the trial. Grossly, they had mild eyelid petechiation and splenic enlargement, along with proventricular haemorrhage in 2 birds.[101] Histologically, mild inflammation and necrosis was seen in the eyelid, along with lymphoid necrosis in a number of tissues. The brains showed non-suppurative perivascular cuffing. It was found that lesions were equally distributed between the cerebrum and the medulla oblongata with no immunohistochemical staining detected in any tissues (presumably because virus had been cleared before the birds were euthanased at day 10).[101]

2.8 The molecular basis for pathogenicity

2.8.1 Defining pathogenicity

Whilst the terms virulence and pathogenicity are sometimes used interchangeably, it is helpful to define them when attempting to determine their basis. Pathogenicity can be thought of as the ability of the organism to cause damage or disease in a host.[105, 106] Pathogenicity is therefore determined by a combination of factors, such as those attributed to the pathogen, the host and the environment. As a result, the pathogenicity of an organism may vary depending on the host species, or the immune status of the host. Virulence on the other hand, has been defined as a measure of the degree of pathogenicity and as such is a more quantitative factor when measured in a known host in a laboratory setting. In the context of laboratory research, virulence can be measured in a number of ways such as the median lethal dose (LD_{50}), or by survivability indexes.[107] It is often described as a physical characteristic of the pathogen in question, attributed to certain virulence factors such as the toxin produced by a bacteria or a viral surface protein.[106] In addition, virulence can be used in an epidemiological context to ascribe increased case fatality rates at the population level due to a particular, more virulent pathogen.[105] Certain characteristics of a virulent pathogen may exert an effect in some

hosts whilst causing little pathology in others. For example, a virulent virus as defined by an experimental model may be pathogenic in poultry but not in waterfowl.

Therefore, both virulence and pathogenicity must be examined in the context of the host with factors such as host immune status being particularly important. Obviously, there will be significant overlap between factors that cause increased pathogenicity and virulence with certain virulence factors contributing to increased pathogenicity in a particular species. Whilst the focus of this research will be the pathogenicity of NDV, standard nomenclature around ND uses the terms 'virulent' and 'avirulent' to classify virus strains. As such, 'virulent' and 'avirulent' will be used when referring to the OIE classification.

2.8.2 Assessing pathogenicity

A number of techniques have been used to define and quantify the pathologenicity of ND viruses in vivo, such as the intracerebral pathogenicity index (ICPI), the intravenous pathogenicity index (IVPI) and the mean death time in eggs (MDT).

The IVPI scores clinical signs in intravenously inoculated 6 week old chickens, whereas the ICPI uses a 0-2 scoring system of intracerebral inoculation of 1 day old chicks. The MDT measures the average time of embryonated eggs to die after allantoic inoculation with virus.[21] The ICPI was previously the most commonly used and well validated of the pathogenicity tests, however it has a number of drawbacks in that it represents an artificial route of inoculation and also has significant welfare concerns. The World Organisation for Animal Heath (OIE) has acknowledged this and recommends that there must be strong justification for the use of the ICPI over *in-vitro* methods.[6] As a consequence, sequencing of the fusion protein cleavage site has replaced the ICPI in the majority of laboratories for determining the pathogenicity of a virus. However, as with all diagnostic tests, pathogenicity tests are not perfect and in some cases these tests have not accurately reflected the virulence seen in the field cases. For example, a number of studies have shown that viruses with similar ICPIs have had variable pathogenicity experimentally, when using a natural route of infection.[108, 109]

Additionally, sequencing of the fusion protein cleavage site of some NDV isolates has also not always correlated with pathogenicity.[86, 104, 108, 110] In some cases, mesogenic viruses, which contain virulent, multibasic cleavage sites, when inoculated into poultry in an

experimental setting, may only produce mild clinical signs.[97] This is also the case with some instances of pigeon paramyxovirus infection of poultry, where virulent cleavage sites have not been reflected in increased pathogenicity indexes or clinical signs in field infections.[111]

Finally, NDVs with avirulent F gene cleavage sites have been shown to have ICPI and IVPI indices characteristic of virulent NDV strains.[112] Table 2.2 contains previously published pathogenicity indices from some NDVs and shows that some of various indices do not always correlate with the cleavage site motif, as indicated by the PPMV-1 and SQZ/04 viruses.

Virus	Fusion protein cleavage site	Virulence*	MDT	ICPI	IVPI	Reference
La Sota	GRQGRL	Avirulent	103	<0.5	0.0	[97]
Roakin	RRQKRF	Virulent	68	1.45	0.0	[97]
SQZ/04	GRQGRL	Avirulent		2.00	2.68	[112]
PPMV-1 340/91	RRQKRF	Virulent		0.66	0.22	[111]
Texas GB	RRQKRF	Virulent	55	1.75	2.7	[97]
Herts 33	RRQRRF	Virulent	49	1.9	2.7	[97]

*Table 2.2 A comparison of pathogenicity indices for a range of NDVs (MDT: velogenic <60 hours, mesogenic 60-90 hours, lentogenic >90 hrs; ICPI: velogenic >1.5, mesogenic 0.7-1.5, lentogenic <0.7; IVPI: range 0 – 3 with virulent viruses approaching 3; *virulence as determined by the fusion protein cleavage site motif)*

2.8.3 Viral determinants of pathogenicity

The pathogenicity of NDV in poultry varies widely between strains of the virus. This variation was recognised very early in the history of NDV with some strains causing high mortalities in chickens compared with others that caused only mild respiratory disease. Determining the molecular basis for pathogenicity and virulence is an important step in both diagnostics and research and helps to identify strains that are likely to cause severe disease so that control measures can be instituted.

Over the last couple of decades, the molecular basis for the virulence and pathogenicity of NDV has been studied in detail, predominantly due to advances in molecular technology and the use of reverse genetics. Throughout the literature, molecular studies have primarily focused on the fusion (F) and haemagglutinin-neuraminidase (HN) genes due to

their key roles in virus entry into cells. However, in a comprehensive review by Dortmans et al., other virulence factors for ND were discussed.[3] It was noted that whilst the F protein cleavage site is a key determinant of virulence, additional factors such as the viral replication complex play important roles in the pathogenicity of the virus. The roles of each of the NDV proteins in pathogenicity are reviewed below.

Fusion protein

The fusion protein has long been recognised as the primary determinant of virulence for NDV. The ability of host proteases to cleave the precursor F_0 glycoprotein into its active form is particularly important. Activation of the F_0 protein into the F_1 and F_2 polypeptides allows cell fusion to occur and viral entry into the host cell.[113] The ability of proteases to cleave the glycoprotein is dependent upon the pathotype of the virus. Cleavage of the precursor protein in lentogenic ND viruses can only be achieved by certain trypsin-like enzymes, which restricts the activity of the virus in the host to particular cells and organ systems, primarily epithelial cells of the respiratory and gastrointestinal tracts.[114] However, cleavage of the precursor protein in velogenic viruses can be achieved by multiple cellular enzymes, allowing viral entry into numerous tissues and the potential for widespread pathology in multiple organ systems. These enzymes include furin and PC6 and their functionality is dependent on the amino acid sequence of the viral cleavage site.[115] Molecular analysis has shown that for furin-like proteases to cleave the protein, there is a requirement for multiple basic amino acids at the F_0 cleavage site, along with a phenylalanine at residue 117 (the N terminus of the F_1 protein).[116, 117] The OIE's definition of a virulent NDV reflects these findings.[6]

The significance of this F protein cleavage site has also been substantiated using reverse genetics techniques.[8, 118, 119 120] In a number of studies, when mutating the cleavage site of a lentogenic strain to a virulent motif, the ICPI was shown to dramatically increase albeit not to the same level of a virulent strain with the same cleavage site.[119, 121] In one pathogenesis study, inserting a virulent cleavage motif into the lentogenic backbone only very marginally increased antigen distribution in 4 week old chickens with no mortalities seen.[110] A similar study by the same investigator showed an increase in clinical disease due to the presence of a virulent F gene.[108]

However, there have also been reports of ND viruses isolated from clinically healthy poultry, which have subsequently been found to contain virulent F protein sequences.[122, 123] In addition, in flocks with mild disease and minimal mortalities attributed to infectious bronchitis virus, velogenic ND viruses have also been isolated.[124] In the context of some African viruses with multibasic cleavage sites and minimal pathogenicity, a Q114R mutation was found to attenuate replication.[125]

The pigeon paramyxovirus is also a clear example of where the fusion protein cleavage site does not always reflect pathogenicity for poultry.[126, 127] In one case it was found that two PPMV-1 viruses with different intracerebral pathogenicity indexes (0.025 and 1.3) had only four amino acid differences in their genome.[128] Of these, only a substitution at position 453 from S to P in the F protein was found to effect pathogenicity and changed the ICPI from 1.6 to 1.3 in a Herts backbone.

In 2011, PPMV-1 was detected for the first time in Australia. When this virus was experimentally inoculated into chickens, despite containing a cleavage site of RRQKRF, no clinical disease was observed (Bergfeld, J., unpublished).
This means that whilst the F gene is very important in pathogenicity, it is not the sole determinant. In total there are 20 papers that have investigated the role of the fusion protein in NDV virulence, either alone, or in combination with other proteins, using reverse genetics. Nine of these papers showed a significant association between a virulent F protein cleavage site and pathogenicity. However, three papers showed equivocal associations.

Haemagglutinin-Neuraminidase protein

The HN protein has also been studied to determine its contribution to the pathogenicity and virulence of NDV. There is a strong correlation between HN gene length and pathogenicity in chickens. The HN length varies between 571 and 616 amino acids depending on the NDV strain and longer HN genes are often referred to as having extended open reading frames of between 6 to 45 aa. Viruses with an HN length of 571 aa are solely velogenic, whereas longer HN precursor lengths of 616 aa (45 aa extension) are only found in avirulent viruses. However HN lengths of 577 aa can be found in multiple NDV pathotypes, see Table 2.3.[121]

Virus	HN length (aa)	HN extension (aa)	Pathotype	Reference
Herts 33	571	0	Velogenic	[129]
Anhinga	571	0	Velogenic	[130]
Texas GB	577	6	Velogenic	[131]
Beaudette C	577	6	Mesogenic	[132]
La Sota	577	6	Lentogenic	[130]
B1	577	6	Lentogenic	[130]
Meredith (AUS)	580	9	Velogenic	[133]
Jilin/01/2008	582	11	Velogenic	[134]
Ulster	616	45	Avirulent	[135]
V4	616	45	Avirulent	[130]

Table 2.3 HN lengths of selected ND viruses and their respective pathotypes

The association of virulence with the length of the HN gene may be due to the requirement for cleavage of the HN_0 precursor in avirulent viruses. Viruses with 616 aa HN proteins require cleavage by proteases to become biologically active, as in the case of the Ulster strain.[136] It has been found that without cleavage, the extended C-terminus blocks the sialic acid binding site of the NA domain of the HN protein.[137] This cleavage is not required by velogenic strains such as Texas GB and Herts 33.

Römer-Oberdorfer et al. investigated the role of the HN and F proteins using reverse genetics and found that the F protein cleavage site is the predominant pathogenicity determinant regardless of the HN protein length.[121] However they did note that when an F protein cleavage site of a lentogenic virus was mutated to a velogenic cleavage site, the corresponding virulence of the virus only increased to a mesogenic (not velogenic) level, indicating a potential role in virulence for the HN and/or other proteins. Changing the length of the HN extension in the presence of the virulent F gene did not alter the ICPI significantly, see Table 2.4.

Other studies using similar techniques of interchanging HN genes within a known NDV backbone have produced variable results. In one study the insertion of the HN gene from a mesogenic virus into a lentogenic backbone increased the pathogenicity of the recombinant virus as expected, inducing wider antigen distribution in embryonic tissues and an increased ICPI from 0.00 to 0.75.[138] However, the insertion of a lentogenic HN into a mesogenic backbone did not produce a lentogenic virus; the ICPI decreased from 1.58

to 1.02 with a corresponding increase in the MDT from 62 hrs to 84 hrs. However, a very similar experiment using the same cloned viruses could not repeat these findings though.[108]

Another study used the LaSota backbone with the F cleavage site mutated to a virulent motif to investigate the impact of interchanging segments of the genome with those from the virulent Herts virus.[129] Inserting the entire Herts HN gene into the LaSota mutant increased the ICPI slightly from 1.28 to 1.40. This study also investigated the roles of the head and stem regions of the HN protein in pathogenicity by creating mutants containing the stem of La Sota and the globular head of Herts and vice versa. The resultant ICPIs did not vary significantly, indicating that both the stem and head are involved in virulence.

It had been identified that there was a need for a cysteine residue at position 123 in the HN protein for the formation of disulfide-linked HN dimers.[139] Mutant clones with and without this amino acid showed that the cysteine residue in the HN increased the ICPI of the recombinant virus from 1.28 to 1.49.[140]

Wakamatsu et al. showed that a lentogenic HN could decrease pathogenicity in a Beaudette C backbone, however a virulent HN could not increase the severity of clinical disease in 4 week old chickens.[108] Estevez however, was not able to show any effect of the HN gene on pathogenicity via inoculation of day old chicks intranasally and intraocularly.[141]

Studies on the length of the HN extension have showed that longer extensions leading to an HN length of 616 aa are associated with mild attenuation, although an effect on tissue tropism was not detected.[142] Another investigation of the effect of the HN extension length on pathogenicity showed moderate attenuation with a 45 aa extension, particularly with an R596C mutation.[143] Zhao et al. also found a similar decrease in pathogenicity when a 45 aa extension was added to a mesogenic Anhinga backbone, producing extended MDTs and lower ICPIs compared with the parental strain.[130]

Site-directed mutagenesis has also been used to further understand the role of HN glycosylation sites. As expected, the mutations of glycosylation sites of the HN protein attenuated the associated viruses.[144] Mutations of the HN at position 526 from Y to Q also

attenuated the recovered virus.[132] However, there was a minimal effect on virulence by deleting amino acids associated with the cytoplasmic tail of the HN.[145]

In addition, a study was conducted to observe the effect of mutating the untranslated regions of the NDV HN gene. Results suggested that the 3' end did not have observed effects on virulence, however deletion of the 5' end attenuated the virus *in-vivo*.[146]

Molecular techniques have not yet been used to analyse the effect of the 9 aa extension of Australian ND viruses. However, based on the literature, it is possible that this extension may have an attenuating effect on virulence, albeit not to the same extent as a 45 aa extension.

In summary, 19 papers have investigated the HN protein's role in pathogenicity including some that focus on the length of the HN gene, particular the C-terminal extension. Overall, 5 papers showed a mild to moderate association of the HN protein with pathogenicity, however 3 cases showed no HN effect, remaining papers showed indeterminate results.

Viral replication complex

The viral replication complex comprises the nucleocapsid protein (NP), phosphoprotein (P) and large polymerase protein (L). It has been hypothesized that increased viral replication may increase viral virulence, therefore these proteins alone or in combination may play key roles in the pathogenicity of NDV.[147] There are 2 main studies which have investigated the viral replication complex in detail using reverse genetics.[12, 13] In the study by Rout et al., the proteins were examined individually and it was found that the N and P gene play a minimal role in pathogenicity, however the L gene significantly increased replication. However, interestingly, in this study it was the insertion of a lentogenic L gene that caused greater replication.

The study by Dortmans et al. used the PPMV-1 strain AV324 and the virulent Herts 33 virus to investigate the role of the viral replication complex and the M protein.[12] This work showed the most significant changes in virulence when multiple genes were interchanged simultaneously as measured by the ICPI. Insertion of the NP, P and L genes from the virulent Herts virus into the AV324 backbone increased ICPI from 0.10 to 1.30. This is the

largest change in ICPI that has been produced experimentally with genes other than the F gene. In this study, the matrix protein did not appear to alter pathogenicity significantly.

Another study which implicates the P and L genes in pathogenicity involved the serial passage of an avirulent PPMV-1 virus in chicken brains with an increase of ICPI from 0.44 to 0.9 by passage 5. This was associated with 3 amino acid mutations, 2 in the L protein and 1 in the P.[148]

A further study examining all 6 NDV genes showed that whilst the F gene is the main determinant of pathogenicity, the polymerase L gene is the second most important contributor.[120]

V protein

The V protein is produced by P gene mRNA editing. The V protein may also contribute to the virulence of NDV, by modulating the innate immune response of the host and acting as an alpha interferon antagonist.[10, 149, 150] In one study, mutations introduced into the V protein of recombinant ND viruses were found to attenuate the virus and decrease the virulence of the viruses in embryonated eggs.[151] A further study supported this theory and found that viruses with mutations in the V protein were attenuated *in-vivo*.[149]

Intergenic and untranslated regions

Two papers have investigated the role of the untranslated regions of the NDV genome. The role of the untranslated regions of the HN gene were found to play important roles in pathogenicity, as seen when their deletion at the 5' and 3' ends attenuated the virus.[146] However, mutations of the 3' end did not have any observed effects.

Insertion of a green fluorescent protein gene between the P and M genes did not affect pathogenicity, however insertion of the fluorescent protein gene between the HN and L genes decreased pathogenicity.[152]

Large additions and deletions of the intergenic regions between the F and HN genes and the HN and L genes all attenuated pathogenicity.[153]

2.8.4 Summary

Approximately 42 papers and 9 theses have investigated the molecular basis for pathogenicity of NDV with particular reference to the use of cloning techniques and reverse genetics. Of these papers, 20 focus on the F gene, 19 on the HN gene, 1 on the M gene, 4 on the viral replication complex (NP, P and L), 3 on the V gene and 3 on the intergenic or untranslated regions with a number of papers examining multiple genes at the one time. Some of these mutant viruses can be seen in Table 2.4.

The majority of these reverse genetics studies used the mesogenic Beaudette C as a plasmid backbone so that both increases and decreases in pathogenicity could be detected. Assessment of pathogenicity after mutating a viral clone was commonly achieved using the ICPI, MDT and to a lesser extent IVPI. There were only a small number of studies (4) that used grown chickens to assess tissue tropism and clinical signs with an additional two studies using day old chickens infected by a natural route.

Notably, the majority of papers that have used reverse genetics to assess virulence or pathogenicity have used ICPI and MDT to quantify the effects of their mutations. There is limited work assessing pathogenicity via tissue tropism, survival curves or clinical signs in older birds. The effect of gene mutations on tissue tropism has been evaluated in 3 papers using embryos and one day old chicks inoculated by natural routes of infection.[138, 141, 142] There are only 4 papers that have used birds older than one day.[110] [108] [109, 146] Three of the papers used 4-week-old birds and assessed clinical signs, however the paper by Yan et al used 2-week-old birds only to assess virus distribution in tissues. Therefore, this lack of experimentation using older birds limits the ability apply the research findings, particularly given that the ICPI and MDT do not always correlate with pathogenicity in a field situation.

In summary, mutation of lentogenic fusion protein cleavage sites to virulent cleavage sites markedly increased ICPIs, which suggests that the F gene is a key pathogenicity determinant in most cases. However a number of studies have also shown that virulence of NDV is a result of multiple genes and not solely due to the cleavability of the F gene or the HN gene.[109, 110, 119, 121, 138] The overall consensus therefore is that whilst the amino acid sequence at the fusion protein cleavage site is a major predictor of the ability of an NDV to cause disease, pathogenicity of NDV is a multigenic trait.

Plasmid	Virus	MDT	ICPI	IVPI	Clinical signs	Reference
NDFL	LaSota (lentogen)	-	0	-	-	8
NDFLtag	LaSota with a virulent F gene	-	1.28	-	-	
rNDV	Clone 30 (lentogen with 6aa HN extension)	-	0.0	-	-	121
rNDVF1	Clone 30 with virulent F cleavage site mutation	-	1.28	-	-	
rNDVHN1	Clone 30 with avirulent F cleavage site and 1aa HN extension	-	0.04	-	-	
rNDVH2U	Clone 30 with avirulent F cleavage site and 45aa extension	-	0.19	-	-	
rNDVF1H1	Clone 30 with virulent F cleavage site and 1aa HN extension	-	1.3	-	-	
rNDVF1N2U	Clone 30 with virulent F cleavage site and 45aa extension	-	1.21	-	-	
rBC	Beaudette C (mesogen)	-	1.58	1.45	-	119
rLaSota	LaSota (lentogen)	-	0.00	0.00	-	
rLaSota V.F	LaSota with same cleavage site as BC	-	1.12	0.00	-	
FL-Herts	Full length velogenic Herts	56	1.54	-	-	126
rgAV324	PPMV1	110	0.10	-	-	
FL-Herts (F)^{AV324}	Herts with PPMV1 F gene	52	1.56	-	-	
rgAV324(F)^{Herts}	PPMV1 with Herts F gene	-		-	-	
rLaSota	LaSota	96	0.00	-	-	138
rLaSo BC HN	LaSota with Beaudette C HN	84	0.75	-	-	
rBC	Beaudette C	62	1.58	-	-	
rBC LaSo HN	Beaudette C with LaSota HN	72	1.02	-	-	
LaSota E13-1	Wild type LaSota	125	0.01	0.01	0 sick	110
NDFL	Recombinant LaSota	123	0.08	0.00	0 sick	
NDFLtag	LaSota with a virulent cleavage site	77	1.60	1.71	nasal discharge (1/10)	
rLaSo	LaSota	>90	0.19	0.00	-	108
rBC	Beaudette C	48	1.66	2.06	-	
rLaSoVF	LaSota with virulent cleavage site	59	1.69	2.39	8/10 sick	
rLaSo BCHN	La Sota with Beaudette C HN	>90	0.00	0.00	0 sick	
rBCLaSoHN	Beaudette C with LaSota HN gene	60	1.58	1.27	0 sick	
rAnh	Anhinga (mesogen)	-	0.89	-	-	109
rAnhTkHN	Anhinga with HN from Turkey North Dakota (neurotropic velogen)	-	1.00	-	-	
rAnhTkFHN	Anhinga with F&HN from Turkey ND	-	1.16	-	Head twitch (1/6)	
rAnhCAHN	Anhinga with Calfornia HN (viscerotropic velogen)	-	0.86	-	Depressed (3/6)	
rAnhCAFHN	Anhinga with California F&HN	-	1.14	-		
FL-Herts	Herts 33	-	1.54	-	-	12
FL-Herts (NP-P-L)^{AV324}	Herts 33 with PPMV NP, P and L genes	-	0.55	-	-	
rgAV324	PPMV1	-	0.10	-	-	
rgAV324(NP-P-L)^{Herts}	PPMV1 with Herts NP, P and L genes	-	1.03	-	-	

Table 2.4 A summary of recombinant viruses and their pathogenicity indices. (- , not described)

CHAPTER 3
GENERAL MATERIALS AND METHODS

3.1 Laboratory animal use

Experiments requiring the use of animals were undertaken in accordance with the Australian National Health and Medical Research Council's Australian code of practice for the care and use of animals for scientific purposes.[154] The design of such experiments was examined and approved by the CSIRO Australian Animal Health Laboratory's Animal Ethics Committee.

3.2 Cell culture

DF-1 cells, a continuous chicken fibroblast cell line from the American Tissue Culture Collection (ATCC, CRL-12203), were grown in DMEM Dulbecco's modified Eagle Medium (Life Technologies) with 10% foetal calf serum (FCS; Serana, Bunbury), 2 mM glutamine, amphotericin B (1.25 µg/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were incubated at 37^{o}C with 5% CO_2.

3.3 Virus titration in eggs

Prior to use, viruses were titrated in specific pathogen free (SPF) embryonated eggs (Charles River Laboratories, Australia) and the infectivity titre determined. Viruses were diluted using tenfold dilutions to create a dilution series from 10^{-1} to 10^{-10}. Eggs were inoculated into the allantoic sac with 100 µl of diluted virus per egg, using 4 eggs per dilution. The eggs were then incubated at 37^{o}C for 5 days and the allantoic fluid harvested to test for the presence of virus by haemagglutination.

Haemagglutination activity was determined by adding 50 µl of PBS to each well of a 96-well round bottomed plate. Next, 50 µl of the test allantoic fluid was added to the first column of the plate and diluted across the plate using doubling dilutions for 4 wells. Finally, 50 µl of 0.5% washed chicken red blood cells collected in Alsevers solution was added to each well, including one row each of negative and positive controls. The plates were then left at room temperature for approximately 45 minutes before reading.

Complete haemagglutination was indicated by the absence of a red button at the bottom of the well and samples were described as either positive or negative.

To determine the infective titre of the virus, the Reed and Muench formula was applied and expressed as the 50 percent embryo infectious dose (EID_{50}) per ml.[155]

3.4 Histopathology and immunohistochemistry

Histopathology was performed according to the CSIRO AAHL Standard Operating Procedures (SOP) for the histopathology laboratory (SOP: 13-04-068). All samples were first fixed in 10% in neutral buffered formalin for between 24-48 hours. The samples were trimmed, processed into paraffin wax and sectioned at 4 µm. For histopathology, slides were then stained with haematoxylin and eosin and mounted.

Immunohistochemistry was undertaken using the CSIRO AAHL Immunohistochemistry SOP (SOP: 13-04-096). In all cases, the mouse monoclonal antibody Q91-1 (AAHL) against the NDV nucleoprotein was used as the primary antibody. The DAKO PT LINK (Dako) was used for antigen retrieval by heating the slides to 97^{o}C for 30 minutes then cooling to 70^{o}C in the Envision FLEX Target high pH retrieval solution and washing for 5 minutes in TRIS buffer. After this, endogenous peroxidases were quenched by the addition of a 3 per cent H_2O_2 solution. The tissues were then incubated with a mouse monoclonal antibody (Q91-6, produced at AAHL) against the NDV nucleoprotein at a 1/800 dilution. The EnVision™ FLEX+ Mouse Linker (Dako) was used to amplify the antibody signal prior to the addition of the secondary anti-mouse antibody using the Envision Flex/HRP conjugate and aminoethylcarbazone (AEC) chromogen (Dako). Slides were then counterstained with Lillie-Mayer haematoxylin using 0.5% haematoxylin Certistain (Australian Biostain, Traralgon, Australia) and Scott's tap water before coverslipping with an aqueous mountant.

3.5 Serology

The only serological test used throughout this study was the haemagglutination inhibition (HI) test. The test was performed following OIE procedures with some modifications.[6] The variations from the OIE test method were the use of 8 haemagglutinating units (HAU)

of inactivated homologous antigen instead of 4 HAU and the addition of 0.5% chicken red blood cells instead of 1% red blood cells. Phosphate buffered saline (PBS) and test serum were added in 25 µl volumes, prior to the addition of 25 µl of 8 haemagglutinating units (HAU) of virus antigen. Chicken red blood cells were then added at 0.5% (v/v) in 50 µl. Haemagglutination inhibition was detected after 30-45 minutes of incubation at room temperature via the presence of a red button at the bottom of the well. Samples showing inhibition at a dilution of 1/8 or greater were deemed positive.

Antigen was produced for all four viruses by propagation of each virus in the allantoic cavity of 10-day old embryonated eggs for four days, clarification of allantoic fluid by centrifugation and inactivation by gamma-irradiation at 5 Mrads (50 kGray).

3.6 Virus isolation

Isolation of live virus from tissue samples was undertaken in 9-11 day old SPF eggs. Unless otherwise specified, tissue samples of approximately 0.5 cm^3 were collected into viral transport media containing PBS, penicillin, streptomycin, gentamicin and 1% bovine serum albumin with 2 mm aluminium silicate beads (Biospec Products Inc, Bartlesville OK, USA). The samples were immediately stored at -80^oC until required. Tissue samples were first homogenized for 30 seconds using a bead beater (FastPrep-24, M.P. Biomedicals, Irvine, California, USA). The samples were then centrifuged for 60 seconds at 13,000 rpm with a benchtop microcentrifuge and the supernatant aliquoted. The supernatant was inoculated into the allantoic cavity of the embryonated eggs using 200 µl per egg. If the tissue had already been tested positive via PCR for NDV genome, only 2 eggs were inoculated per sample. If the NDV status of the sample was unknown, 3 eggs were inoculated per sample. The eggs were then incubated at 37^oC for 7 days and were checked daily for any deaths. Before processing, eggs were chilled at 4^oC overnight. The allantoic fluid was harvested and tested for the presence of virus via haemagglutination as described in Chapter 3.3.

3.7 Mean death time in eggs

The mean death time (MDT) of the viruses was determined via inoculation of SPF eggs. Each virus was diluted tenfold to provide four dilutions: 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. Each

dilution was used to inoculate the allantoic cavity of 5, 10-day-old embryonated eggs using 100 µl per egg. The eggs were then incubated at 37ºC. After 8 hours, another 5 eggs were inoculated with the same virus dilutions and also incubated at 37ºC. The eggs were then examined for deaths twice a day for 7 days. The minimum lethal dose was determined as the highest dilution to kill all 10 embryos inoculated with that dilution. Finally, the mean death time could be calculated by taking the average time for the embryos to die when inoculated by the minimum lethal dose.

Classification of the mean death time is represented in Table 3.1.

Pathotype	**Mean Death Time**
Lentogenic	> 90 hours
Mesogenic	60 – 90 hours
Velogenic	< 60 hours

Table 3.1 Mean death time indices

3.8 Nucleic acid isolation

Total RNA was extracted from a number of sources, either tissue samples, allantoic fluid, or cell culture material. Tissue samples were homogenized as described in Chapter 3.6 before inactivation in extraction buffer. Allantoic fluid was first clarified by centrifugation at 10,000 rpm using a Heraeus Multifuge 3SR Plus centrifuge (Thermo Scientific). Cell culture material was inactivated by removing the cell culture media then adding extraction buffer directly to the cells in the tissue culture plate.

Either the MagMAX™-96 Viral RNA Isolation kit (Ambion, Life Technologies, Mulgrave, Australia) or the QIAamp Viral RNA Mini Kit (Qiagen) was used to extract the RNA, in both cases by following the manufacturers' directions and using the extraction buffers provided in the kits.

The MagMAX™ kit was used in conjunction with 96 well deep well plates (Thermo Scientific) and the Kingfisher Flex extraction machine (Thermo Scientific) so that large volumes of samples could be extracted concurrently. Samples were inactivated in MagMAX™ Lysis/Binding solution using 50 µl sample per 130 µl of lysis solution. Wash solution 1 was added to two deep well plates using 150 µl per well. Wash solution 2 was

added to an additional 2 plates, also using 150 µl per well. The elution buffer was added to a standard 96 well plate using 60 µl per well. The samples were also added to a deep well plate along with 20 µl of premixed RNA binding beads (10 µl) and lysis binding enhancer (10 µl). The plates were then loaded onto the Kingfisher machine and the appropriate protocol selected to run.

The QIAamp Viral Mini kit was used when there were less than 20 samples for RNA extraction. The following procedure was used: 560 µl of AVL buffer containing carrier RNA was placed in a 1.5 ml microcentrifuge tube. Then 140 µl of the sample was added to the buffer and vortexed. The samples were incubated at room temperature for 10 minutes and 560 µl of ethanol added to the tube. A QIAamp mini column was then used to collect the virus/buffer mixture in 560 µl aliquots, between which the column was centrifuged at 6000 x g for 1 minute and the flowthrough discarded. Subsequently, 500 µl of buffer AW1 was added and centrifuged at 6000 x g for 1 minute, followed by 500 µl of buffer AW2 with centrifugation at 20,000 x g for 3 minutes. The RNA was then eluted in 60 µl of AVE buffer by centrifugation at 6000 x g for 1 minute. The RNA was either used immediately or stored frozen at -80^{o}C.

3.8.1 Nucleic acid quantification

RNA and DNA samples were quantified using either the NanoDrop spectrophotometer (ND-1000 NanoDrop Technologies, Inc.) or the Qubit fluorometer (Qubit 2.0, Invitrogen).

When using the NanoDrop instrument, the machine was first initialized using 2 µl of nuclease-free water. Following this a blank sample was analyzed using 2 µl of the elution solution that the RNA or DNA was contained in (Qiagen AVE buffer or nuclease-free water). Finally, 2 µl of the sample was added and the sample analysed after selecting the appropriate input (DNA or RNA) on the associated software program.

The Qubit 2.0 was calibrated before use using the supplied Standards #1 and #2 as per the manufacturer instructions, adding 10 µl of standard to 190 µl of working solution. The sample solution was made by adding 1-20 µl of sample to 180-199 µl of working solution such that the total volume was 200 µl. Thin walled, 0.5 ml PCR tubes were used in the instrument.

3.9 Quantitative Polymerase chain reaction (qPCR)

3.9.1 TaqMan RT-PCR

Prior to performing real-time PCR, viral RNA was extracted from samples and quantified according to Chapter 3.8. The reverse transcription and PCR reactions were then performed in one step using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Victoria, Australia), an ABI 7500 real-time PCR machine (Applied Biosystems) and MicroAmp® Fast Optical 96-well Reaction plates (Applied Biosystems). In most cases, the qPCR reaction was a multiplex of the NDV M gene and the 18S rRNA housekeeping gene with primer sequences shown in Appendix 1, Table A1.

The reactions were run using 20 µl volumes containing NDV M gene forward and reverse primers (200 nM of each) and probe (100 nM), along with 18S forward and reverse primers (50 nM of each) and probe (200 nM). Additional reagents included nuclease-free water (5.9 µl), template RNA (2 µl), 2X RT-PCR Buffer (10 µl) and 25X RT-PCR Enzyme Mix (0.8 µl).

Cycling conditions were as follows:
Reverse transcription: 45^{o}C for 10 min
Reverse transcriptase inactivation and denaturation: 95^{o}C for 10 min
Amplification: 45 cycles of 95^{o}C for 15 sec and 60^{o}C for 45 sec

Positive samples were those with a cycle threshold (C_T) less than 40. This threshold has been established as the standard for NDV across the Laboratories for Emergency Animal Disease Diagnosis and Response (LEADDR) Network. Thresholds were set at 0.05 for the NDV M gene and 0.02 for the 18S rRNA gene to allow comparisons to be made across different PCR plates. Reactions were carried out in triplicate with positive, negative and no template control samples used on each plate. If required, copy numbers of the gene of interest were normalized to the 18S rRNA house-keeping gene using standard curves according to Chapter 3.10.

3.9.2 SYBR Green RT-PCR

SYBR Green RT-PCR was used for cytokine expression analysis and viral gene mRNA transcript quantification. The *Power* SYBR® Green RNA-to-C_T™ *1-Step* Kit, ABI 7500 PCR-machine (Applied Biosystems) and MicroAmp® Fast Optical 96-well Reaction plates (Applied Biosystems) were used unless otherwise specified. Reactions were conducted in 10 µl volumes using *Power* SYBR® Green RT-PCR Mix 2X (5.0 µl), forward and reverse primers (200 nM final concentration of each), RT Enzyme Mix 125X (0.08 µl), template RNA (1 µl) and nuclease-free water (3.52 µl). No more than 100 ng of template RNA was used in the reaction. The sequences of the primers used can be found in Appendix 1, Tables A4 and A5.

Cycling conditions were as follows (unless otherwise specified):
Reverse transcription: 48^{o}C for 30 min
Activation of DNA polymerase: 95^{o}C for 10 min
Amplification: 45 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min
Melt curve: 95^{o}C for 15 sec, 60^{o}C for 15 sec, 95^{o}C for 15 sec

A melt curve was performed after each reaction to determine whether there was any non-specific amplification. Samples were analysed in triplicate with negative and no template control samples used on each plate.

The data was analysed using the $\Delta\Delta C_T$ method and expressed as fold changes (relative quantity) compared with the control with 95% confidence intervals calculated using AB 7500 software v2.0.6 (Life Technologies). The efficiencies of the PCR reactions were first determined by standard curve to ensure that the reactions were comparable.

3.10 RNA copy number quantification

Quantification of viral RNA in tissue samples was achieved by generating standard curves to normalise copy numbers of the NDV M gene to copy numbers of 18S rRNA. This allows for comparisons of NDV RNA loads to be made between tissue samples without weighing individual samples.

3.10.1 RNA isolation

18S RNA was extracted from an uninfected control chicken brain sample using the MagMAX™-96 Viral RNA Isolation kit (Ambion) as per Chapter 3.8. Similarly, NDV-M RNA was isolated from allantoic fluid inoculated with the Meredith/02 ND virus.

3.10.2 Conventional Polymerase Chain Reaction

cDNA was generated from the NDV-M gene and 18S fragments and then amplified using the Superscript III One-step RT-PCR kit with Platinum® *Taq* DNA polymerase (Applied Biosystems). The following reagent volumes were used for both gene RNA segments: template RNA (1 µl), buffer 2X (25 µl), forward primer (10 µM, 1.5 µl), reverse primer (10 µM, 1.5 µl), enzyme (1 µl) water (20 µl), for a total reaction volume of 50 µl.
The reactions were run using the following cycling conditions:
cDNA synthesis: 45ºC for 30 minutes x 1 cycle
Denaturation: 94ºC for 2 minutes x 1 cycle
Amplification: 94ºC for 15 seconds, 55ºC for 30 seconds, 68ºC for 60 seconds x 40 cycles
Final extension: 68ºC for 5 minutes x 1 cycle
4ºC ∞

3.10.3 Agarose gel electrophoresis

The PCR products generated from both reactions were then run on a 1% agarose gel for 45 minutes at 100V as per Chapter 3.13.2.

3.10.4 Gel purification

Bands of the appropriate size corresponding to 121 bp for the NDV M gene and 187 bp for 18S were extracted from the gel and the DNA purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) as described in in Chapter 3.13.3.

3.10.5 Ligation

The NDV M gene and 18S cDNA fragments were ligated into the pGEM®-T easy vector (Promega, Madison, USA). The ligation reactions were performed in 10 µl volumes using the following reagents: rapid ligation buffer 2X (5 µl), pGEM®-T easy vector (0.5 µl), T4 DNA ligase (1 µl) and cDNA (3.5 µl). The samples were incubated for 1 hour at room temperature. Both a positive control and background control were used.

3.10.6 Transformation

The vectors were then transformed in *E. coli* cells as per Chapter 3.13.5. The transformed cells were plated out onto LB agar plates containing ampicillin (100 mg/ml) and incubated at 37^{o}C overnight. Colonies were inoculated into centrifuge tubes containing 2 ml LB with ampicillin (0.1 mg/ml) and incubated once again at 37^{o}C for 12-16 hours.

3.10.7 Colony PCR

The colonies selected for broth inoculation above were also screened via PCR according to Chapter 3.13.6 using the GoTaq® Hot Start Green Master Mix. M13 universal forward and reverse primers were used (10 µM, 1 µl), the primer sequences of which can be found in Appendix 1, Table A2.
The resulting PCR products were then run on a 1% agarose gel at 100V for 45 minutes to check for bands of the appropriate size.

3.10.8 Plasmid purification

If the colony PCR showed that the vector with the correct insert had been obtained, the corresponding broth that had previously been incubated overnight was used to purify the plasmid DNA using the Wizard® Plus SV Minipreps DNA Purification System (Promega). The method is described in Chapter 3.13.7. The plasmid DNA was eluted into 100 µl of nuclease-free water and quantified as previously in Chapter 3.8.1, using the NanoDrop. The DNA was stored frozen at -20^{o}C until required for sequencing. Sanger sequencing was used to ensure that no sequence mutations had been introduced into the insert DNA (NDV-M gene or 18S) as per Chapter 3.11 using M13 primers (Appendix 1, Table A2).

3.10.10 Maxiprep

After ensuring that the NDV-M and 18S rRNA plasmids contained the correct DNA sequences, the plasmids were grown to a high concentration using the PureYield™ Plasmid Maxiprep System according to Chapter 3.13.7, using 200 µl ampicillin to grow the plasmids in the LB broth.

3.10.11 Digestion

The circular plasmid DNA was linearised by restriction enzyme digestion. The reactions were carried out in 50 µl volumes using 20 µl of the NDV-M or 18S rRNA plasmid DNA,

buffer B 10X (5 µl, Promega), SpeI restriction enzyme (2 µl, Promega), bovine serum albumin (5 µl, BSA) and nuclease-free water (18 µl). The digestion mixture was incubated for 37ºC for 90 minutes.
The digested products were run on a 1% agarose gel as above to remove any undigested products. Bands of the expected size, 3,136 bp for NDV-M and 3,200 for 18S were excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) as described previously (Chapter 3.13.7).

The resulting linearised DNA was quantified using the NanoDrop to determine the DNA concentration in the elution.

3.10.12 Copy number determination

In order to create a standard curve for each of the gene targets, it was necessary to calculate the number of DNA molecules per µl. It had been previously shown in our laboratory using Hendra and Nipah viruses (also single strand RNA viruses), that calculating standard curves using DNA was equivalent to the curves created using RNA, therefore reverse transcription of the DNA into RNA was not undertaken (Glenn Marsh, pers. comm.). The weight of DNA per µl was used in the calculation, thereby calculating the copy number per µl.

The calculation was as follows:
Number of molecules (copy number) =
[weight (ng) * 6.0221 x 10^{23*}]/[(plasmid length + insert (bp)) * 660 Da * 1 x 10^{9}]
*Avogadro's number

3.10.13 Standard curves

Standard curves were created using the plasmid cDNA that had previously been isolated from the control chicken brain sample (18S rRNA) or the Meredith/02 virus infected allantoic fluid (NDV-M). Both RNA samples were diluted ten-fold in nuclease free water to create a dilution series from neat to 10^{-7}. A qPCR reaction was then performed using the reagents and conditions specified in Chapter 3.9.1. The C_T values were plotted against the dilutions which had been converted into copy numbers and the line of best fit calculated (Microsoft Excel).

The line of best fit equation was as follows:
y = a*ln(x) + b,
where y = C_T, x = copy number, a = slope of the curve, b = the y intercept

Therefore, copy numbers could then be calculated from C_T values using the line of best fit as below:
Copy number (x) = e^((Ct – b)/a)

3.10.14 Normalisation of qPCR data

The NDV M gene copy numbers were then normalised to a defined number of 18S rRNA copies. This was undertaken so that comparisons between different tissue samples could be made and to take into account the fact that different sized samples with different tissue densities were used in the RNA isolation step. Initially, the average copy number of the sample run in triplicate was taken and then NDV M gene copy numbers were normalised to 10^8 copies of 18S rRNA.

In most cases, the data were then also log transformed to allow for a better visual representation of the spread of the results.

3.11 Sanger sequencing

Sequencing of plasmids and short fragments of viral genome was undertaken using the Sanger sequencing method. The sequencing PCR reaction was performed using M13 primers (pGEM-T easy vector) or pCAGGS primers (pCAGGS vector) and the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). A list of primers can be found in Appendix 1 (Tables A2 and A3).

Reactions were undertaken in 20 µl using template DNA (1 µl), BigDye® 5X sequencing buffer (3 µl), BigDye® ready reaction mix (1 µl), forward primer and nuclease-free water.

The cycling conditions were as follows:
Denaturation: 96ºC for 1 minutes x 1 cycle
PCR amplification: 96ºC for 10 seconds (denaturation), 50ºC for 5 seconds (annealing),

60°C for 4 minutes (extension) x 25 cycles
4°C ∞

Sequencing was then performed using an ABI 3130xl (or 3500xl) Genetic Analyzer (Applied Biosystems). When the 3130xl machine was used, 1 ng/100 bp of template was used compared with 2 ng/100 bp of template for the 3500xl machine.

3.12 Whole Genome Sequencing

Whole genome sequencing was performed using the MiSeq (Illumina) platform based on a previously published method.[156]

3.12.1 Virus purification

Initially, viruses were grown in the allantoic cavity of 10-day-old SPF eggs. The harvested allantoic fluid was initially clarified by centrifugation at 10,000 rpm to remove gross debris material using a Heraeus Multifuge 3SR Plus centrifuge (Thermo Scientific). A discontinuous sucrose gradient was then prepared using 50% (w/v) sucrose in 2 ml at the bottom of an ultracentrifuge tube (Beckman Coulter SW41) and 20% sucrose in 4 ml at the top of the tube. The sucrose solutions were prepared by adding the appropriate amount of sucrose powder (Sigma) to Tris-NaCl-EDTA (TNE) buffer (i.e. 10 g sucrose in 40 ml of TNE for a 20% solution). The 50% solution was first added to the centrifuge tube. Secondarily, the 20% solution was added by slowly pouring down the side of the tube, making sure that the interface was not disrupted. The interface between the two solutions was then marked for easy identification after centrifugation. Finally, 5 ml of the clarified allantoic fluid was slowly added to the tubes. The tubes were centrifuged for 3 hours at 36,000 rpm for 90 minutes at 4°C in a JS-24 centrifuge (Beckman Coulter) with a slow acceleration and deceleration applied so that the gradient was not disturbed. After centrifugation, the cloudy band containing purified virus that had formed at the interface between the sucrose solutions was removed and 1 ml was stored at -80°C until required.

3.12.2 RNA isolation

RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen) as per Chapter 3.8, however vacuum was used rather than centrifugation. Initially, 1 ml of purified allantoic fluid was added to 4 ml of AVL buffer (without carrier RNA) and vortexed

for 15 seconds. The samples were then incubated at room temperature for 10 minutes. A vacuum connector was then connected to the Eluator™ Vacuum Elution Device (Promega) and a QIAamp Mini spin column added. A vacuum was applied and the allantoic fluid/buffer solution was added to the spin column in 630 µl aliquots. The column was then washed using 750 µl of AW1 buffer followed by 750 µl of AW2 buffer. Next, the column was placed in a wash tube and centrifuged at 14,000 rpm for 1 minute using a benchtop centrifuge and the filtrate discarded. Following this, the column was positioned in a 1.5 ml Eppendorf tube and the RNA incubated with 60 µl of AVE buffer for 1 minute. Finally, the RNA was eluted by centrifugation at 8,000 rpm for 1 minute and stored frozen and -80^{o}C.

3.12.3 Reverse transcription

Viral RNA from the previous step was reverse transcribed using the Superscript III transcriptase kit (Invitrogen). Initially, 1 µl of 454 cDNA primer containing random octamers was added to 1 µl of dNTP Mix (10 mM, Invitrogen) with 11 µl of template RNA (details of the cDNA primer can be found in Appendix 1, Table A2). The mix was heated at 65^{o}C for 5 minutes and placed on ice for 1 minute. Next, 4 µl of 5X first-strand buffer was added, along with 1 µl 0.1 M DTT, 1 µl RNaseOUT (Invitrogen) and 1 µl Superscript III. The tubes were then flicked to mix the reagents and incubated at 50^{o}C for 1 hour and then at 100^{o}C for 2 minutes. Samples were left to cool on ice.

3.12.4 Double strand synthesis

Double strand synthesis was performed by adding 20 µl of the sample to 2.5 µl 10X DNA polymerase buffer (Promega M195A), 1 µl dN8 454 cDNA primer and 1.5 µl Klenow (Promega M220A). The samples were mixed and incubated at 37^{o}C for 30 minutes, then inactivated at 70^{o}C for 15 minutes.

3.12.5 Random PCR

The cDNA was amplified via random PCR using the Expand High Fidelity kit (Roche). Reactions were undertaken in 50 µl volumes using 5 µl 10X Expand Buffer with $MgCl_2$, 1 µl dNTP (10mM), 4 µl 454 amplification primer (20 µM), 37 µl nuclease free water, 1 µl Expand High Fidelity enzyme and 2 µl template cDNA.
All samples were amplified in duplicate using the reaction conditions:

Denaturation: 95^{o}C for 5 minutes x 1 cycle
PCR amplification: 94^{o}C for 1 minute (denaturation), 50^{o}C for 1 minute (annealing), 72^{o}C for 1 minute (extension) x 25 cycles
Final extension: 72^{o}C for 7 minutes x 1 cycle
4^{o}C ∞

The sequence of the 454 amplification primer can be found in Appendix 1, Table A2.

3.12.6 Check gel

In order to determine whether the PCR products contained amplicons of the expected size, they were run on a 1% agarose gel as per Chapter 3.13.2. The products were expected to form a streak on the gel between 300 – 800 bp. The samples used for sequencing were all greater than 300 bp.

3.12.7 PCR product purification

The PCR products containing amplicons of the correct size were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The procedure was performed according to the method described in Chapter 3.13.3, however the original PCR products were used as opposed to excising the gel. Therefore the initial procedure involved adding an equal amount of membrane binding solution to the PCR product before adding the mixture to the SV column assembly.

3.12.8 Removal from the AAHL secure laboratories

In order to access the Illumina MiSeq, the PCR products were removed from the AAHL secure laboratories. This required the products to be decontaminated by adding ethanol such that 70% of the total volume was 100% ethanol.
After removal, the DNA was quantified using the Qubit DS high sensitivity DNA assay (Life Technologies).

3.12.9 Sequencing

Sequencing of the DNA was performed using the MiSeq platform (Illumina) according to the manufacturer's instructions. The genome of each of the four viruses was assembled using read mapping and *de novo* assembly using the CLC Genomics Workbench (CLC Genomics Workbench v.6.0.5). Read mapping was performed for the Meredith/02, PR/98

and Texas GB viruses using previously published sequences AY935490, AY935497 and GU978777. Whereas the alignment for the Herts 33/56 virus consisted of 2 independent read mappings and 2 denovo assemblies. For the purposes of this research the ends of the sequences were not required and therefore not determined.

3.13 Cloning (P and V genes)

3.13.1 Conventional PCR

The Superscript III One-step RT-PCR kit with Platinum® *Taq* DNA polymerase (Applied Biosystems) or the Q5® High-Fidelity 2X Master Mix (New England BioLabs) was used to amplify cDNA. Reagents were used according to the kit manufacturer's instructions unless otherwise specified. The PCR reactions were performed using a Multigene Gradient thermal cycler (Labnet). Annealing temperatures were adjusted according to the NEB online Tm calculator (http://tmcalculator.neb.com/#!/).

3.13.2 Agarose gel electrophoresis

In most cases, unless otherwise stated, a 1% agarose gel was used to separate DNA fragments. The 1% gel was prepared using agarose powder (Sigma) in tris-acetate-edta (TAE) buffer with the addition of 0.01% SYBR® Safe (Invitrogen). Samples were loaded onto the gel with 6X gel loading buffer (Thermo Scientific) and a 1 Kb Plus DNA ladder (Invitrogen) and were electrophoresed for 45 minutes at 100V. Images of the resulting bands were taken using a DC120 camera (Kodak).

3.13.3 Gel purification

The Wizard® SV Gel and PCR Clean-Up System (Promega) was used to purify DNA bands after agarose gel electrophoresis. Bands were cut from the gel, weighed and placed into 1.5 ml microcentrifuge tubes. Membrane binding solution was added at 10 µl per 10 g of gel and the tube then incubated at 60^{o}C and vortexed until the gel had dissolved. The dissolved gel was then transferred to an SV minicolumn combined with a collection tube. The tubes were centrifuged at 13,000 rpm for 1 minute and the flowthrough discarded resulting in the DNA binding to the SV minicolumn. The column was washed twice with membrane wash solution and centrifuged at 13,000 rpm for 1

minute after the first wash then for 5 minutes after the second wash. The DNA was eluted in 50 µl of nuclease-free water after centrifugation.

3.13.4 Ligation

Ligation reactions were incubated at room temperature for one hour or at 4ºC overnight.

3.13.5 Transformation

All vectors were transformed using Top 10 F *Escherichia coli* (*E. coli*) cells using 2 µl of the ligated plasmid and 50 µl of the *E. coli* cells which had previously been thawed on ice. The mixture was transferred to a cuvette and the cells electroporated at 1.8 Volts using a Gene Pulser (Bio-rad). Into the cuvette, 500 µl of Luria-Bertani broth (LB) was added and mixed. The mixture was then transferred to a 15 ml tube and incubated for 1 hour at 37ºC on a platform shaker at 250 rpm. An aliquot, of the transformed mixture, 50 µl, was then plated out using beads onto LB agar plates containing an antibiotic appropriate to the plasmid. The plates were incubated at 37ºC overnight. Colonies were then picked from the plates using toothpicks and added to 15 ml centrifuge tubes containing 2 ml LB with the appropriate antibiotic (0.1 mg/ml) and incubated overnight at 37ºC on a platform shaker at 250 rpm. The same colonies were simultaneously used in a screening colony PCR to ensure that the vector contained the correct target insert.

3.13.6 Colony PCR

Colony PCRs were conducted with the GoTaq® Hot Start Green Master Mix by placing the colonies collected via toothpicks directly into the mastermixes. The reactions were undertaken in 10 µl volumes containing GoTaq Master Mix 2X (5 µl), forward primer (10 µM), reverse primer (10 µM) and nuclease-free water to make a total reaction volume of 10 µl.

Cycling conditions were:

Denaturation: 95ºC for 2 minutes x 1 cycle

PCR amplification: 95ºC for 30 seconds (denaturation), 50ºC for 30 seconds (annealing), 72ºC for 60 seconds (extension) x 30 cycles

Final extension: 72ºC for 7 minutes x 1 cycle

4ºC ∞

3.13.7 Plasmid purification

Plasmids were purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega) or the PureYield™ Plasmid System (Promega).
An aliquot (1 ml) of the LB broth was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 5 minutes to form a cell pellet. The supernatant was removed and the pellet resuspended in cell resuspension solution (250 µl). Alkaline protease (10 µl) was added to the mix and incubated for 5 minutes at room temperature. Neutralization solution (350 µl) was added next and the mixture centrifuged at 13,000 rpm for 10 minutes. The solution was then poured into a spin column which had been inserted into a collection tube in order to bind the DNA to the column. The tubes were centrifuged at 13,000 rpm for 1 minute and the flowthrough discarded. Wash solution containing ethanol (750 µl) was added to the column and centrifuged once again. The wash was repeated with 250 µl of wash solution. Finally, the DNA was eluted into 100 µl of nuclease-free water.

For greater yields of plasmid DNA, the PureYield™ Plasmid Maxiprep System (Promega) was used. The 2ml plasmid broths obtained during the transformation step above (Chapter 3.13.5) were used to grow the subsequent culture by adding 1 ml aliquots into 200 ml LB with appropriate antibiotics in 1 litre conical flasks. The flasks were incubated overnight at 37^{o}C on a platform shaker at 250 rpm. The cultures were centrifuged at 5,000 x g for 10 minutes and the supernatant discarded. The pelleted cells were suspended in 12 ml cell resuspension solution. Next, 12 ml of cell lysis solution was added and the solution incubated for 3 minutes at room temperature. After adding 12 ml of neutralization solution, the lysed cells were centrifuged at 14,000 x g for 20 minutes. The lysate was then poured through the PureYield™ clearing columns into the PureYield™ Maxi binding columns using the Eluator™ Vacuum Elution Device (Promega). The binding columns were washed using 5 ml of endotoxin removal wash and 20 ml of column wash, in both cases by applying a vacuum to the columns. The plasmid DNA was eluted by vacuum into 1 ml of nuclease-free water.

The resultant DNA concentration was analysed using the NanoDrop (Chapter 3.8.1) and the DNA sequenced using Sanger sequencing (Chapter 3.11).

3.14 Transfection

3.14.1 Transfection with plasmid DNA

Transfection of DF-1 cells with plasmid DNA was performed using Lipofectamine® LTX with Plus™ Reagent (Invitrogen). Cells were seeded at a density of 5 x 10^4 cells per well in 24-well tissue culture plates and grown until 80% confluency using growth media with 5% foetal calf serum. Cells were then transfected using a DNA:Lipofectamine ratio of 1:3 after previously optimising the ratio to achieve maximum transfection efficiency. Initially, growth media was removed and the cells washed once with PBSA. Subsequently, 500 µl of Opti-MEM (ThermoFisher Scientific) was added to all wells. Next (in per well volumes), in separate tubes, Lipofectamine LTX (1.5 µl) was diluted in Opti-MEM (25 µl) and plasmid DNA (0.5 µg) was diluted in Opti-MEM (25 µl) with PLUS reagent (0.5 µl). The diluted reagents were incubated for 5 minutes and then the diluted DNA was added to the diluted Lipofectamine LTX and incubated for 5 minutes. The lipid complex (50 µl) was then added to the cells in a dropwise manner and the cells incubated at 37^{o}C with 5% CO_2. After 10 hours, the lipid complex was removed from the cells and growth media with 5% foetal calf serum added.

The transfection optimisation process was conducted using four different ratios of DNA to Lipofectamine (1:2, 1:3, 1:4 and 1:5) in Opti-MEM, using the pCAGGS-GFP plasmid in DF-1 cells. Each of these dilutions were added to cells in duplicate. One of the duplicates was allowed to incubate overnight in Opti-MEM and the other had the media removed after 6 hours and replaced with growth media. The transfection efficiency was assessed at 24 and 56 hours by counting the number of fluorescent cells per four 10X fields. The optimum transfection conditions were found to occur when the transfection media was removed after 6 hours and with a 1:3 DNA to Lipofectamine ratio.

3.14.2 Transfection with polyionsinic:polycytidylic acid (Poly I:C)

Poly I:C was also used to transfect cells in order to mimic infection with viral dsRNA. Growth media was removed from cells and washed with PBSA. Then the same volumes of Lipofectamine LTX, Opti-MEM complex were used as in Chapter 3.14.1 with 0.5 µg of Poly I:C added instead of plasmid DNA.

3.15 Primers and probe design

In-house primers and probes were designed using Geneious (Geneious R8, v. 8.0.5) software and were manufactured by Geneworks at their PCR/Sequencing purity level. Lyophilized primers and probes were made up into 100 µM stock solutions using nuclease-free water. Aliquots of 20 µl were then diluted to 10 µM working stocks and stored frozen at -20ºC. These aliquots were only thawed a maximum of 3 times or until degradation was evident.
A complete list of primer and probe sequences can be found in Appendix 1.

3.16 Sequence Analysis

The two software programs used to analyse both Sanger sequence and whole genome sequence data, were the CLC Genomics Workbench (CLC Genomics Workbench v.6.0.5) and Geneious (Genious R8, v. 8.0.5).

3.17 Statistical analysis

Statistical analyses were undertaken using either GraphPad Prism 5.02 for Windows (GraphPad Software) or MS Excel 2013 (Microsoft).

CHAPTER 4

AN AUSTRALIAN NEWCASTLE DISEASE VIRUS WITH A VIRULENT FUSION PROTEIN CLEAVAGE SITE PRODUCES MINIMAL PATHOGENICITY IN CHICKENS

4.1 Introduction

Newcastle disease (ND) is a viral disease of birds, which is endemic in many countries and can have severe impacts on domestic poultry and in some cases wild birds. It is caused by virulent strains of Newcastle disease virus (NDV), which is classified within the genus *Avulavirus* in the family *Paramyxoviridae* and is synonymous with avian paramyxovirus serotype 1 (APMV-1).[21] NDV is a negative sense RNA virus, containing 6 genes (3'-NP-P-M-F-HN-L-5') that encode 7 proteins: the nucleocapsid protein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin-neuraminidase protein (HN), the large polymerase protein (L), and the V protein which is produced by RNA editing of the P gene.[157] There is wide variability in pathogenicity between isolates of NDV which has led to grouping of the various viruses into five pathotypes (from most pathogenic to least pathogenic): viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic.[21,6] Viscerotropic velogenic viruses are highly pathogenic with lesions associated with the gastrointestinal tract. Neurotropic velogenic viruses are also highly pathogenic, however respiratory and neurological signs predominate. Mesogenic viruses vary in their pathogenicity but often present with respiratory signs in younger birds and low morbidity and mortality. Lentogenic viruses are associated with mild respiratory disease, and birds infected with asymptomatic viruses show little to no clinical signs at all.[6] The intracerebral pathogenicity index (ICPI) is commonly used to classify these viruses and is conducted by intracerebral inoculation of 1 day old chicks. In addition, sequencing of the F protein cleavage site has also been found to correlate with pathogenicity.[129] According to the OIE guidelines, virulent, notifiable strains are those which contain multiple basic amino acids (lysine or arginine) at the F protein cleavage site with a phenylalanine at residue 117, or have an intra-cerebral pathogenicity index (ICPI) of 0.7 or greater.[6] Therefore, both mesogenic and

velogenic viruses are all classified as virulent despite some mesogenic viruses causing minimal disease.[3, 126]

From 1998, Australia experienced multiple outbreaks of ND in commercial poultry including broiler and layer chickens and quail, before it was subsequently eradicated in 2002.[56,63] Whilst outbreaks of ND have not occurred in Australia since 2002, NDVs continue to circulate in wild birds and outbreaks of pigeon paramyxovirus (APMV-1 genotype VIb) have occurred in pigeons and wild birds from 2011, with the potential to cause disease in poultry.[158] Currently circulating avirulent viruses in wild birds in Australia are from both Class I and Class II genotype I and whilst it was speculated that the 1998-2002 outbreak viruses may have originated in wild birds, testing of wild birds at the time of the outbreak was not able to definitively prove this.[53, 159, 160]

The ND outbreaks in 1998 were predominantly located within the Mangrove Mountain region of New South Wales (NSW) and were immediately preceded by the isolation of a NDV in chickens from Peats Ridge, NSW (PR/98). PR/98 was detected in association with respiratory disease in broiler chickens and was shown to have an avirulent F protein cleavage site.[53] It was found that the sequence of the PR/98 virus cleavage site varied by only two nucleotides from the sequence found in virulent viruses. These two nucleotide changes induced two amino acid changes from ^{112}RRQGRL^{117} (PR/98) to ^{112}RRQRRF^{117} (virulent).[53] The PR/98 virus was thought to be the precursor to the virulent viruses detected during this time, as it was isolated on the majority of properties that went on to have outbreaks of ND.

Whilst most of the ND viruses isolated from the 1998-2002 disease outbreaks were classified as virulent on account of their F protein cleavage site motifs, it was noted that the disease syndrome often differed from what was expected of a velogenic virus.[9, 56] There was slower spread throughout the flock, less severe clinical signs and a lower case fatality rate than would be observed with typical velogenic ND viruses. Of particular note was an outbreak in 2002 on a layer farm in Meredith, Victoria, in which egg production had decreased by 40%, production of soft-shelled eggs had increased and there was a very slight rise in mortalities per month from 0.4% to 0.8%.[9] Despite not having the clinical appearance of a velogenic virus, the ICPI of the isolated NDV was determined to be 1.61

and the F protein cleavage site motif was ^{112}RRQRRF^{117}.[71] As a result, a stamping out policy was instituted and the birds were culled.

Given the significant trade and economic implications of ND outbreaks, it is important to determine whether the current OIE definition of virulent, notifiable strains of NDV based on the F protein cleavage site motif is justified. A number of studies have shown that whilst the F protein is critical in predicting the behaviour of an ND virus, pathogenicity is multifactorial and proteins other than the F protein may play key roles.[12, 109, 120]

There have been limited *in vivo* experimental studies using Australian ND virus isolates. Previous experimental studies using an Australian virus with a virulent F protein cleavage site of ^{112}RRQRRF^{117}, isolated from Glenorie, NSW (9809-19-1107) found that affected birds showed mild to severe depression, respiratory disease and neurological signs with no mortalities and recovery after 10 days.[101, 104]

Although the field data indicate that some Australian viruses isolated from 1998-2002 with virulent cleavage sites are not highly pathogenic, it is not understood why there is this apparent contradiction. Given the paucity of information on the disease from these outbreaks, one of our objectives was to confirm the field observations that chickens infected with these viruses develop only mild clinical disease. A second objective was to determine the pathogenesis of the viral infections in chickens evaluated experimentally.

In this study we characterised the infection caused by a virulent Australian NDV (Meredith/02) and compare it with an avirulent Australian virus (PR/98). Two highly virulent reference viruses, the viscerotropic Herts 33/56 and neurotropic Texas GB were included for comparison. The results of this work will be important in understanding the risk framework associated with Australian NDVs, the appropriate outbreak response to their detection in poultry flocks and whether the current OIE definition of ND is applicable in the Australian situation.

4.2 Materials and Methods

All of the animal experiments included in this paper were conducted in accordance with the Australian National Health and Medical Research Council's Australian Code of Practice for

the Care and Use of Animals for Scientific purposes.[154] The design of the experiments and care of the animals were approved by the CSIRO Australian Animal Health Laboratory's Animal Ethics Committee.

All experimental work involving the use of live virus was conducted at biosafety level 3 (BSL-3).

4.2.1 Animals and handling

Fifty, 8-week-old specific-pathogen-free (SPF), White Leghorn chickens were used in the study. The birds were randomly assigned to one of five groups of ten birds (including one control group). Each group of chickens was housed in a separate room at BSL-3 comprising bare floor and a retreat area with artificial perches and sawdust. The male to female ratios of the birds in each group varied from 1:1 to 2:3. The birds were allowed four days to acclimatise to the rooms before challenge and had free access to feed and water at all times. Animals were monitored twice daily when clinically healthy and up to every two hours during the day when clinical signs were evident. Prior to euthanasia, birds were anaesthetised with a combination of xylazine at 5 mg/kg (Xylazil-20; Ilium, Smithfield, Australia) and ketamine at 53 mg/kg (Metamil; Ilium, Smithfield, Australia) delivered via the intramuscular route.

4.2.2 Virus isolates

Four ND virus isolates were used in this study; two Australian viruses and two virulent viruses exotic to Australia (Table 4.1). The Meredith/02 virus was originally isolated from a cloacal swab collected during an outbreak of ND at Meredith, Victoria in 2002 and was classified as virulent because it contained a polybasic F protein cleavage site and an ICPI of 1.61.[71] The PR/98 virus was isolated at Peats Ridge, NSW in 1998 and is known as the avirulent precursor virus to the subsequent virulent ND outbreaks. The PR/98 virus has an ICPI of 0.6.[71] Both of these Australian viruses had undergone one passage in eggs prior to their use in this study. The two exotic viruses were Herts 33/56, which is a viscerotropic velogenic virus (Class II, genotype IV) and Texas GB (Class II, genotype II), which is a neurotropic velogenic virus with ICPIs of 1.9 and 1.75 respectively.[97] Both viruses were imported from Central Veterinary Laboratory, Weybridge, England in 1992 with an unknown passage history.

Prior to use, each virus isolate was grown in 9-11 day old embryonated SPF chicken eggs (Charles River Laboratories, Australia) via inoculation of 0.2 ml into the allantoic cavity and incubation at 37^{o}C for 4 days.

Virus name	Reference number	Location	Date of isolation	Fusion protein cleavage site motif[a]
Meredith/02	02-1334/ 0205-10-0004	Meredith, Victoria, Australia	8/05/2002	^{112}**RR**Q**RR**F^{117}
PR/98	98-1154/ 9809-04-1555	Peats Ridge, New South Wales, Australia	14/09/1998	^{112}**RR**QG**R**L^{117}
Herts 33/56	9303-11-1630	Hertsfordshire, UK	1933	^{112}**RR**Q**RR**F^{117}
Texas GB	9302-26-1330	Texas, USA	1948	^{112}**RR**Q**KR**F^{117}

Table 4.1 Virus isolates used in the study. [a]Basic amino acids are indicated in bold

4.2.3 Experimental design

Each group of ten birds was challenged with one of the four virus isolates using allantoic fluid diluted in PBS to give a titre of 10^5 50% embryo infectious doses (EID_{50}) in 200 µl per bird. The inoculum was equally divided between the ocular, nasal and oral routes and administered into the conjunctival sac, both nares and caudal pharynx respectively. The control birds were inoculated similarly with 200 µl of phosphate buffered saline. At days 2 and 4 post challenge (dpc), two birds from each group were euthanized to assess viral replication in tissues. The remaining six birds were then observed over 14 days. Birds were euthanized at the point at which they developed moderate clinical signs or at the end of the trial period on day 14. Moderate clinical signs were defined as inactivity, infrequent eating, huddling and not reverting to normal behaviour on stimulation.

Prior to challenge and at 7 days post challenge, 1-2 ml of blood was collected from the ulnar vein and placed into serum separator tubes. At euthanasia, blood was collected via cardiac puncture after anaesthesia. The blood was then allowed to clot at room temperature for 30 mins before being centrifuged at 1300 g for 10 mins. The serum was

removed and stored frozen at -20ºC until testing. Immediately prior to testing, sera were heat inactivated at 56ºC for 30 minutes.

At euthanasia a range of tissue samples were collected from all birds. Samples for histology and immunohistochemistry were fixed in 10% neutral buffered formalin for 24-48 hours before processing. Tissues for histology included trachea, lung, air sac, heart, spleen, eyelid, thymus, caecal tonsils, bursa, pancreas, proventriculus, gizzard, duodenum, skin, skeletal muscle and brain. For molecular analysis and virus isolation, approximately 0.5 cm^3 samples of fresh brain, spleen, lung, kidney and small intestine, were collected into tubes containing 970 µl viral transport media (PBS, penicillin, streptomycin and gentamicin and 1% bovine serum albumin) with 2 mm aluminium silicate beads (Biospec Products Inc, Bartlesville OK, USA). These samples were stored frozen at -80ºC until required. Tissues from control birds were only tested by histopathology and immunohistochemistry.

4.2.4 Serology

The titre of antibodies was measured by haemagglutination inhibition (HI) assay using standard methods, as described in Chapter 3.5.[6]

4.2.5 Histopathology and Immunohistochemistry

Histopathology and immunohistochemistry were performed according to Chapter 3.4, using a mouse monoclonal antibody (Q91-1) for immunohistochemistry.

4.2.6 RNA isolation and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA isolation and qRT-PCR was conducted as described in Chapters 3.6, 3.8 and 3.9. RNA was extracted using the MagMAX™-96 Viral RNA Isolation kit (Ambion) with 100 µl of the tissue supernatant combined with 260 µl MagMAX™ Lysis/Binding solution. After isolation, RNA was quantified via NanoDrop, as per Chapter 3.8.1 and used immediately with the remainder stored at -80ºC.

qRT-PCR in triplicate was conducted using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Victoria, Australia). The NDV M gene primers and probe were multiplexed with eukaryotic 18S primers and probe, the sequences of which can be found in Appendix 1, Table A1. Previously published NDV M gene primers and probe were multiplexed with eukaryotic 18S primers and probe to allow for quantitation of NDV RNA copy numbers (Table 2).[161]

Relative quantification was conducted via standard curves generated according to Chapter 3.10. NDV M gene copy numbers were then expressed relative to 10^8 copies of 18S RNA.

4.2.7 Virus Isolation

Virus isolation was only conducted on PCR-positive tissue samples. An aliquot (200 µl) of the tissue homogenate supernatant was inoculated into the allantoic sac of 10 day old SPF embryonated chicken eggs in duplicate, which were then incubated at 37°C for 7 days. Allantoic fluid was then tested for haemagglutination activity (HA).

4.2.8 Sequencing

The whole genome of each of the virus isolates was sequenced via the MiSeq (Illumina) platform using a variation of a published method which can be found in Chapter 3.12 [156].

At the conclusion of the trial, partial re-sequencing of the virus isolates was performed on RNA extracted from tissue samples to ensure that the F protein cleavage site and the HN extension had not mutated during *in vivo* replication. The sequencing was performed by Sanger sequencing using published primers[7, 71], the Big Dye Terminator Reaction Kit v. 3.1 (Applied Biosystems) and an ABI 3130xl Genetic Analyzer (Applied Biosystems) according to Chapter 3.11.

4.2.9 Statistical Analysis

Kaplan-Meier survival curves were generated using GraphPad Prism 5.02 for Windows (GraphPad Software, San Diego, USA). Survival curves were analysed using the Mantel-

Cox log-rank test with pairs of survival curves compared at a time. GraphPad Prism was also used to analyse serology and PCR data using the Student's t-tests and the Mann-Whitney U test respectively. Statistical significance was set at $p<0.05$ for all analyses.

4.3 Results

4.3.1 Clinical signs

All birds challenged with the Meredith/02 virus survived until the end of the trial (apart from the clinically healthy birds euthanized at 2 and 4 dpc according to the study design). However at 3 dpc, the five birds were seen to be slightly less active in the morning with two birds displaying very mild laboured respiration. These clinical signs had resolved within 6 hours.

No clinical signs were observed in any of the birds inoculated with the PR/98 virus.

All birds inoculated with the exotic Herts 33/56 and Texas GB viruses showed moderate clinical signs as defined by a decreased response to stimulation, decreased feed intake and huddling before the end of the trial period and were euthanized at that point. Eight birds in the Herts 33/56 group showed clinical signs at 2 dpc, including moderate depression, head tucking and an unwillingness to move away when approached. All birds in this group were then euthanized at 2 dpc, including 2 healthy birds as per the study design. Two birds inoculated with the Texas GB isolate showed moderate depression, head tucking and mild ataxia at 4 dpc and were euthanized at that time. At 5 dpc, the remainder of these birds (n=4), displayed ataxia, with three birds showing an obvious head twitch. These birds were then euthanized at 5 dpc.

No abnormalities were detected clinically in any of the control birds.

A survival curve comparing the euthanasia time points for birds infected with each of the different viruses is given in Figure 4. The survival times for the birds infected with the Australian viruses were significantly different from those infected with the Herts 33/56 ($p=0.0009$) and Texas GB viruses ($p=0.0012$).

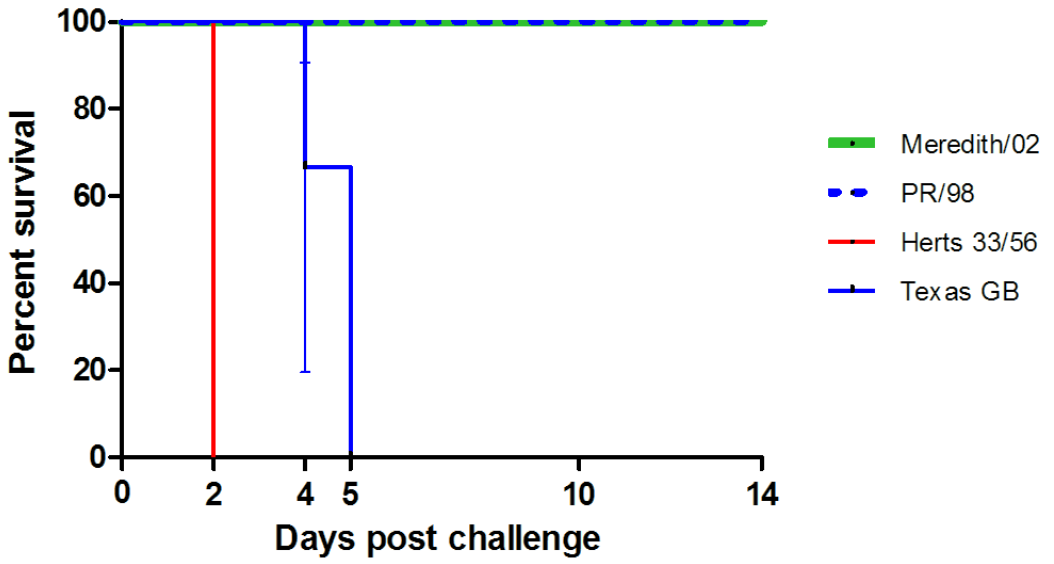


Figure 4.1 Kaplan-Meier survival curve of the birds infected with the four viruses over 14 days. The plot does not include the 4 birds in each group that were euthanased on 2 and 4 dpc challenge according to the study design. Error bars indicate 95% confidence intervals.

4.3.2 Serology

All birds were negative for serum antibodies prior to challenge. All birds in the Meredith/02 and PR/98 groups had seroconverted by 7 dpc with significantly different (p=0.01) geometric mean titres of $2^{8.2}$ and $2^{6.3}$ respectively. By 14 dpc, mean titres had reached $2^{10.2}$ in the Meredith/02 group and $2^{7.2}$ in the PR/98 group, again showing significant differences (p=0.0004) between the virus strains. None of the birds inoculated with the Herts 33/56 virus had seroconverted due to the early euthanasia time point at 2 dpc. Low levels of antibodies (2^2 and 2^4) were detected at 5 dpc in two birds inoculated with the Texas GB virus with the rest of the birds being euthanized without detectable antibodies.

4.3.3 Gross Pathology

Gross pathological findings were restricted to the birds infected with the exotic isolates of NDV. In birds infected with the Herts 33/56 isolate, the most consistent findings were haemorrhagic caecal tonsils (10/10), haemorrhage at the junction of the proventriculus and oesophagus (4/10) and mild splenic enlargement with pale mottling (4/10). Birds inoculated with the Texas GB isolate displayed moderate cloudiness of the air sacs (2 birds on 4 dpc and 2 birds on 5 dpc), along with one bird with mild splenomegaly on 5 dpc.

There were no gross lesions detected in birds inoculated with the Australian ND viruses or in control birds.

4.3.4 Histopathology

The histological lesions noted in birds infected with the Meredith/02 and PR/98 isolates of NDV were minimal. The lesions involved mild infiltrates of heterophils in the epithelium and submucosa around sites of inoculation, i.e. the nasal turbinates (Fig. 4.2), trachea and conjunctiva. These features were observed in the Meredith/02 group within all birds euthanized on 2 and 4 dpc and in 2 birds euthanized on 14 dpc. Only the two birds euthanized on 2 dpc in the PR/98 groups showed mild heterophilic infiltrates in the nasal turbinates and conjunctiva.

In birds infected with the Herts 33/56 virus, histological lesions were predominantly seen in sites of lymphoid tissue accumulation such as the thymus (10/10), conjunctiva (10/10), spleen (9/9), proventriculus (at the oesophageal and gizzard junctions, 10/10), caecal tonsils (10/10), bursa (9/10) and nasal turbinates (8/10). In these regions, there was moderate to marked necrosis and apoptosis of lymphocytes and macrophages, with oedema, fibrin deposition and heterophil infiltration (Fig. 4.3).

In birds inoculated with the Texas GB virus, there was moderate loss of cilia throughout the tracheas, with occasional necrosis of epithelial cells and moderate numbers of inflammatory cells, predominantly heterophils (8/10). The other significant lesions were seen within the central nervous system and comprised perivascular lymphocyte cuffing and glial nodule formation throughout the cerebrum, cerebellum and brainstem (8/10). Occasional neuronal necrosis was also seen, most commonly in the cerebellum or brainstem. These lesions were seen in all birds except those euthanized on 2 dpc.

There were no histological lesions detected in any of the control birds.

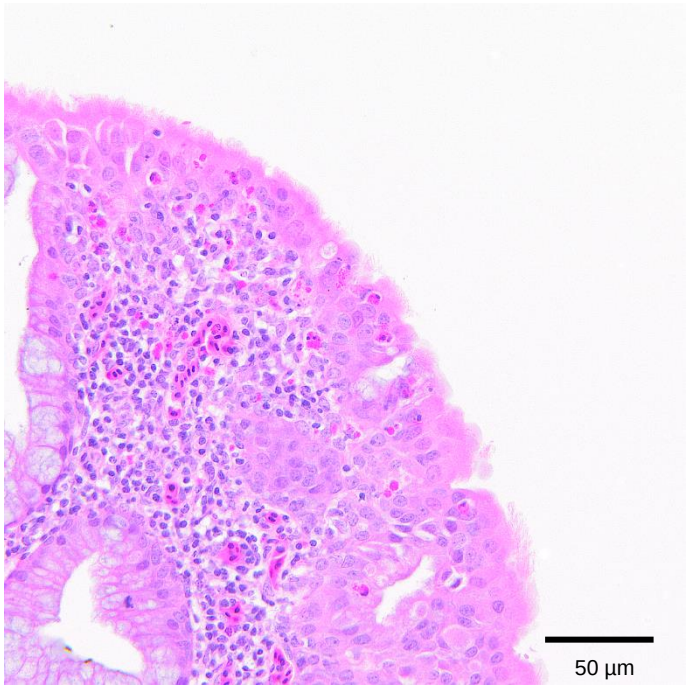


Figure 4.2 Meredith/02, nasal turbinates, mild heterophil and lymphocyte infiltrates in the epithelium and submucosa with loss of cilia. Haematoxylin and eosin.

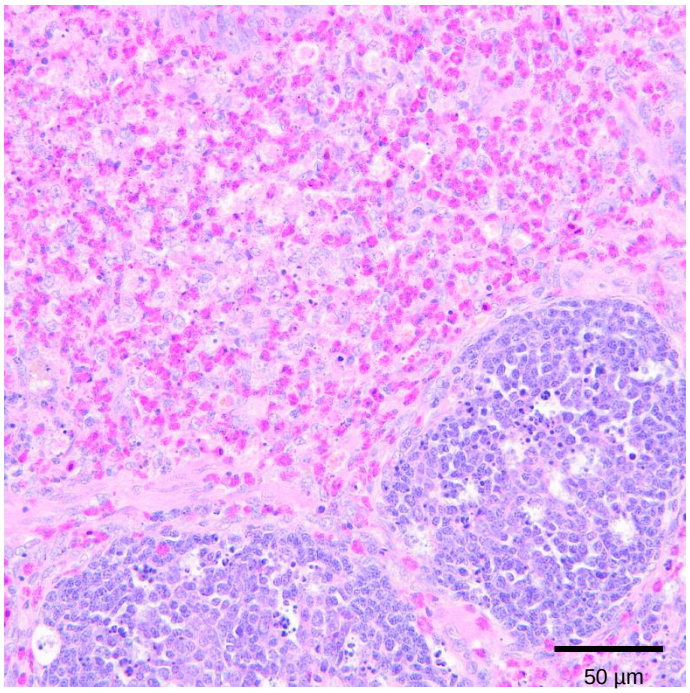


Figure 4.3 Herts 33/56, caecal tonsils, necrosis of lymphoid follicles with a marked heterophil infiltrate. Haematoxylin and eosin.

4.3.5 Immunohistochemistry

Antigen was detected in birds infected with all four viruses, however the distribution and quantity of the staining varied between the different virus groups (Table 4.2). Antigen staining appeared as fine to moderate-sized granules in both the cytoplasm and the nucleus of cells. At euthanasia of the Herts 33/56 birds, antigen was detected in the brain (9/10), nasal turbinates (10/10), eyelids (10/10), larynx/trachea (4/8), lung (10/10), heart 10/10, spleen (9/9), kidney (7/10) and caecal tonsils (10/10). In the Texas GB birds, antigen was also detected in the brain (8/10), nasal turbinates (10/10), eyelids (8/9), larynx/trachea (9/10), lung (8/10), heart (8/10), spleen (10/10), kidney (8/10) and caecal tonsils (8/10). The quantity of antigen was scored on a 3 point scale (Table 4.2). In contrast, birds inoculated with the Meredith/02 virus showed antigen predominantly restricted to sites of inoculation, with staining seen in the nasal turbinates (7/10), eyelid (4/10), larynx/trachea (Fig. 4.5 3/8), spleen (4/4) and caecal tonsils (3/9). Birds inoculated with the PR/98 virus also showed antigen staining in the nasal turbinates (4/10), eyelid (2/10), larynx/trachea (Fig. 4.6, 2/10) only on 2 and 4 dpc. There was also antigen staining in the spleen of one bird euthanized a 14 dpc. The antigen detected in the spleen and caecal tonsils at 2 and 4 dpc in the Meredith/02 birds indicates a greater degree of systemic replication than with the PR/98 virus. No immunohistochemical staining was detected in any tissues from control birds.

Comparisons of the staining intensity between birds infected with the different viruses can be seen in Figure 4.4.

Animal identification	Day of euthanasia	Brain	Brainstem	Nasal Turbinates	Eyelid	Larynx/ Trachea	Lung	Heart	Spleen	Kidney	Caecal tonsils
Meredith/02											
6	2	-	-	+	++	n/d	-	-	+	-	+
7	2	-	-	+	++	n/d	-	-	+	-	-
1	4	-	-	+++	+	+++	-	-	+	-	+
4	4	-	-	+	+	+	-	-	++	-	+
2	14	-	-	+	-	+	-	-	-	-	-
3	14	-	-	+	-	-	-	-	-	-	-
5	14	-	-	+	-	-	-	-	-	-	-
8	14	-	-	-	-	-	-	-	-	-	-
9	14	-	-	-	-	-	-	-	-	-	-
10	14	-	-	-	-	-	-	-	-	-	n/d
PR/98											
13	2	-	-	+	+	+	-	-	-	-	-
19	2	-	-	++	-	-	-	-	-	-	-
12	4	-	-	++	+	+	-	-	-	-	-
16	4	-	-	++	-	-	-	-	-	-	-
11	14	-	-	-	-	-	-	-	-	-	-
14	14	-	-	-	-	-	-	-	+	-	-
15	14	-	-	-	-	-	-	-	-	-	-
17	14	-	-	-	-	-	-	-	-	-	-
18	14	-	-	-	-	-	-	-	-	-	-
20	14	-	-	-	-	-	-	-	-	-	-
Herts 33/56											
21	2	++	+	+	+++	+	++	++	+++	+	+++
22	2	++	+	+	+++	-	+	+	+++	+	+++
23	2	+	-	++	+++	+	+	+	+++	-	+++
24	2	+	-	+	+++	++	++	+	+++	+	+++
25	2	++	++	+++	+++	n/d	++	++	n/d	+	+++
26	2	+	++	+++	+++	-	++	++	+++	++	+++
27	2	++	-	+++	+++	++	+	++	++	-	+++
28	2	+	-	++	+++	n/d	+	+	+++	++	+++
29	2	-	-	+	+++	-	++	++	+++	-	+++
30	2	+	-	+	+++	-	+	++	++	+	++

Texas GB											
12	2	-	-	+	++	-	-	-	+	-	-
20	2	-	-	+	+	++	-	-	+	-	+
11	4	+	+	++	-	+++	++	++	+	++	+
16	4	++	++	+	+	+	++	++	+	++	+
18	4	++	+	++	+	+++	++	++	++	++	++
19	4	+	+	++	n/d	+++	+	+	++	+	+
13	5	++	++	++	+	++	+	+	+	++	+
14	5	++	+	++	+	++	++	++	+	+	+
15	5	+++	+++	+	++	++	+	+	+	+	-
17	5	+++	+++	+	+	+++	++	+	++	++	+

Table 4.2 Tissue staining by immunohistochemistry (n/d: not done, +++ widespread staining, ++ clusters of positive cells, + small number of individual positive staining cells, - no staining)

The cellular tropism also varied between viruses, with birds infected with the Herts 33/56 viruses showing a stronger lymphoid tissue tropism than both the Australian viruses and the Texas GB virus. The birds infected with the Herts 33/56 virus showed a predominance of staining in lymphoid tissue within mononuclear cells in both lymphocytes and macrophages (Fig. 4.7). The positive staining lymphoid tissue was found throughout the upper respiratory tract and gastrointestinal tract. Antigen was also detected in the brain in both neurones and glial cells. The most notable difference between the Texas GB virus and the other viruses was the large amount of staining in the central nervous system, in which cell bodies and processes of large neurons were stained, particularly Purkinje cells in the cerebellum (Fig. 4.8) and brainstem nuclei. These birds also displayed greater staining of respiratory epithelial cells in the upper respiratory tract when compared with the Herts 33/56 infected birds (Table 4.3).

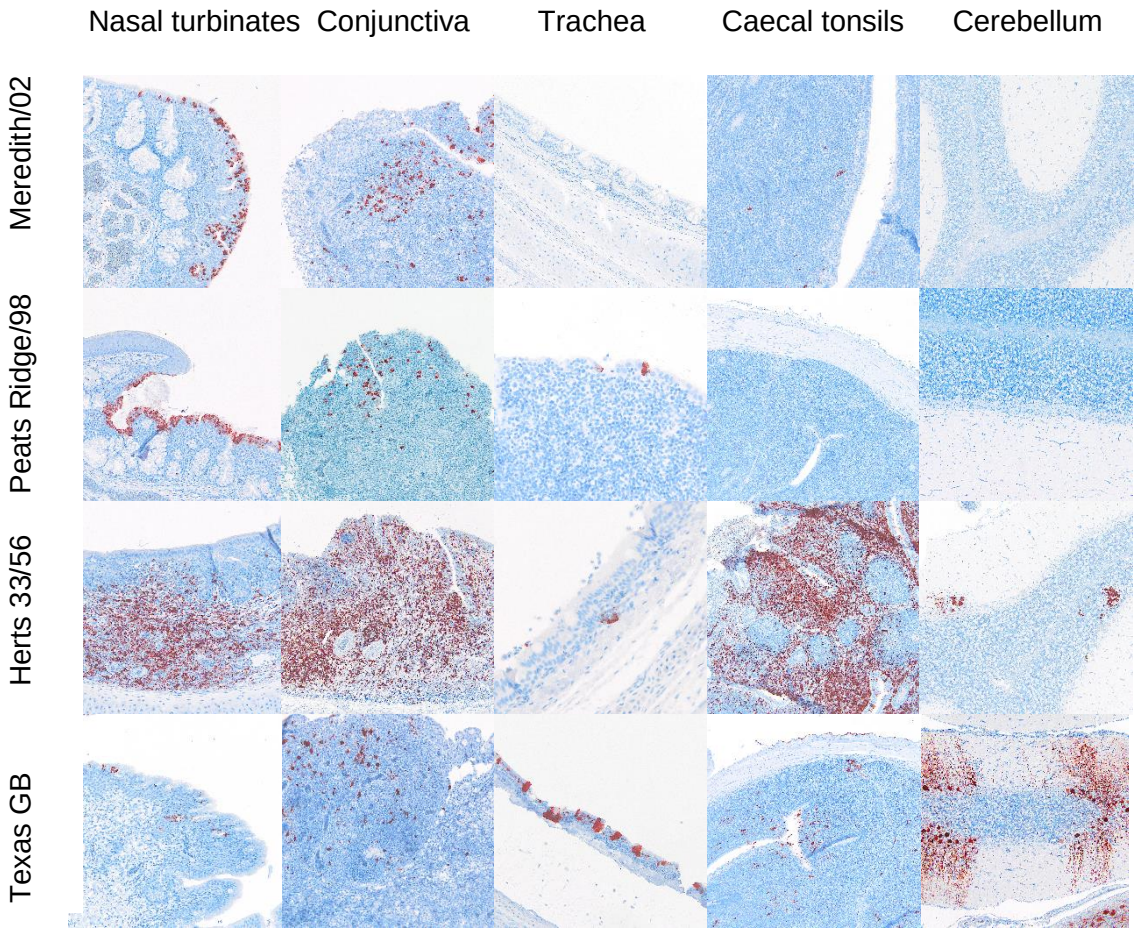


Figure 4.4 Immunohistochemical staining of nasal turbinates, conjunctiva, trachea, caecal tonsils and cerebellum. Immunohistochemistry for NDV nucleoprotein (red).

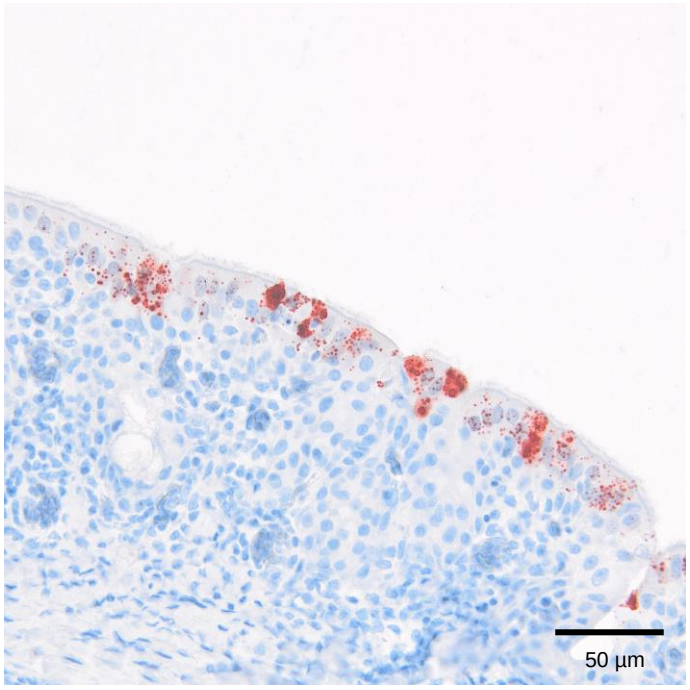


Figure 4.5 Meredith/02, larynx, staining of epithelial cells. Immunohistochemistry for NDV nucleoprotein (red).

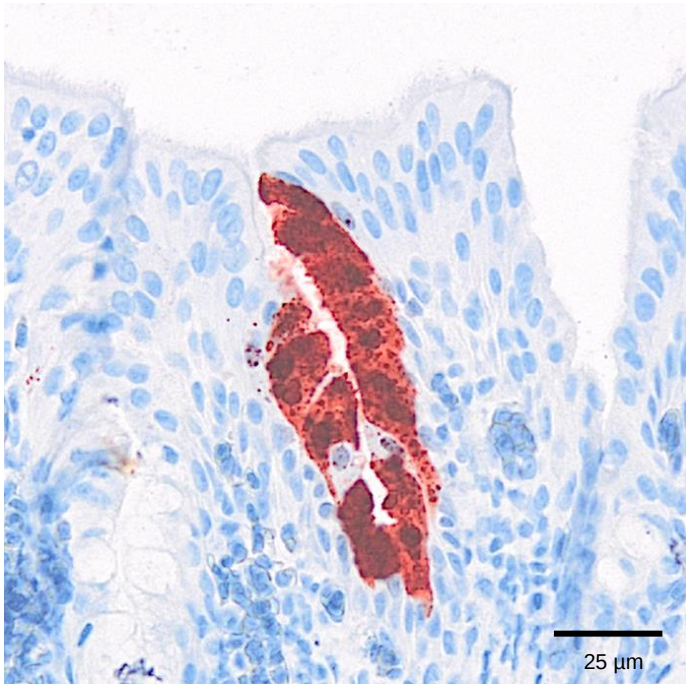


Figure 4.6 Peats Ridge/98, larynx, staining of epithelial cells. Immunohistochemistry for NDV nucleoprotein (red).

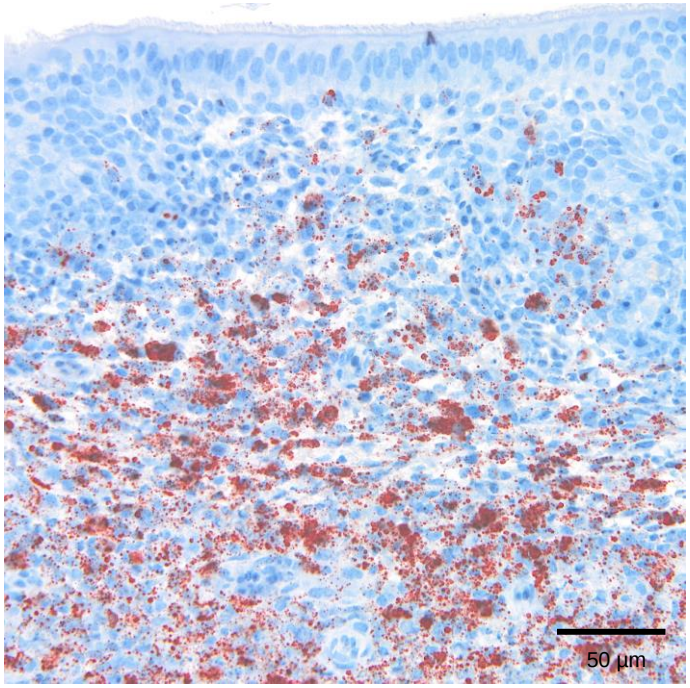


Figure 4.7 Herts 33/56, nasal turbinates, staining of lymphocytes and macrophages. Immunohistochemistry for NDV nucleoprotein (red).

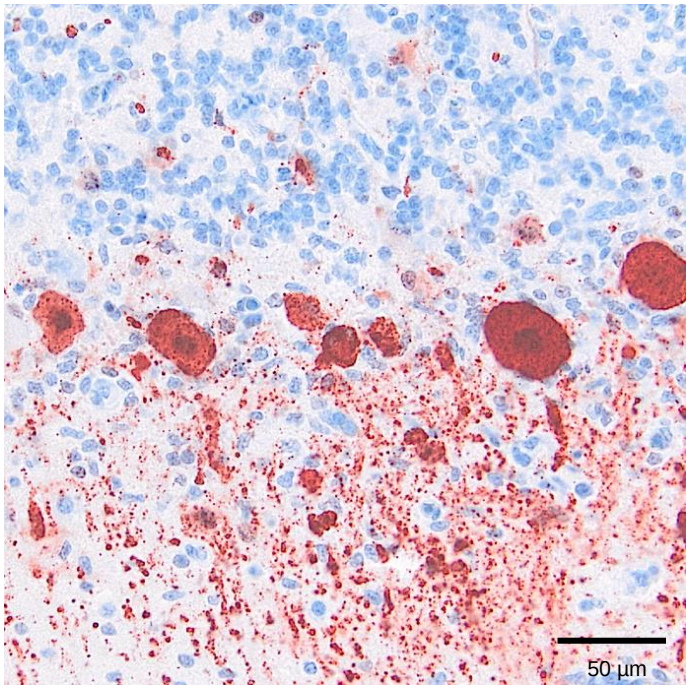


Figure 4.8 Texas GB, cerebellum, staining of Purkinje cells and dendrites. Immunohistochemistry for NDV nucleoprotein (red).

Animal identification	Day of euthanasia	Epithelial	Lymphoid
Meredith/02			
6	2	+	-
7	2	+	-
1	4	+++	-
4	4	+	-
2	14	+	-
3	14	+	-
5	14	-	+
8	14	-	-
9	14	-	-
10	14	-	-
PR/98			
13	2	+	-
19	2	++	-
12	4	++	-
16	4	++	-
11	14	-	-
14	14	-	-
15	14	-	-
17	14	-	-
18	14	-	-
20	14	-	-
Herts 33/56			
21	2	+	+
22	2	-	+
23	2	+	++
24	2	-	+
25	2	++	+++
26	2	+	+++
27	2	+	+++
28	2	+	++
29	2	-	+
30	2	-	+
Texas GB			
12	2	+	+
20	2	+	+
11	4	++	-
16	4	+	-
18	4	++	-
19	4	++	-
13	5	++	-
14	5	++	-
15	5	+	-
17	5	+	-

Table 4.3 Cellular tropism within the nasal turbinates by immunohistochemistry
(+++ widespread staining, ++ clusters of positive cells, + small number of individual positive staining cells, - no staining)

4.3.6 Polymerase chain reaction

Five tissue samples (brain, spleen, lung, kidney, small intestine) from each of the birds were tested for NDV RNA via qRT-PCR (Table 4.4). All five tissue samples from all ten birds infected with the Herts 33/56 virus showed detectable levels of NDV RNA at the time of euthanasia (2 dpc). The majority of tissue samples collected from birds infected with the Texas GB virus at 2, 4 and 5 dpc were positive for NDV RNA (42/50 samples). Initially only the spleen samples from the two birds euthanized on 2 dpc were positive, however all tissues from the remaining birds were positive on both 4 dpc and 5 dpc. A total of 15 samples were NDV RNA positive from the 10 birds inoculated with the Meredith/02 virus, including all 10 spleen samples with one positive kidney sample (4 dpc), three positive lung samples (2 and 4 dpc) and one positive duodenum sample (4 dpc) . Only 5 tissues were viral RNA positive from the birds infected with the PR/98 virus, consisting of two spleen samples from 2 and 4 dpc, two duodenum samples from 2 and 14 dpc and one cerebrum samples from 14 dpc.

When comparing viral RNA loads between the viruses, 2 dpc was chosen as the comparative time point because at the point of euthanasia at 14 dpc, all of the birds infected with the exotic velogenic viruses had already been euthanized. Comparisons of RNA loads in the spleen using the Mann-Whitney U test showed that when compared with each other, all four viruses had significantly different copy numbers at 2 dpc when normalised to 18S ($p<0.05$; Fig. 4.9).

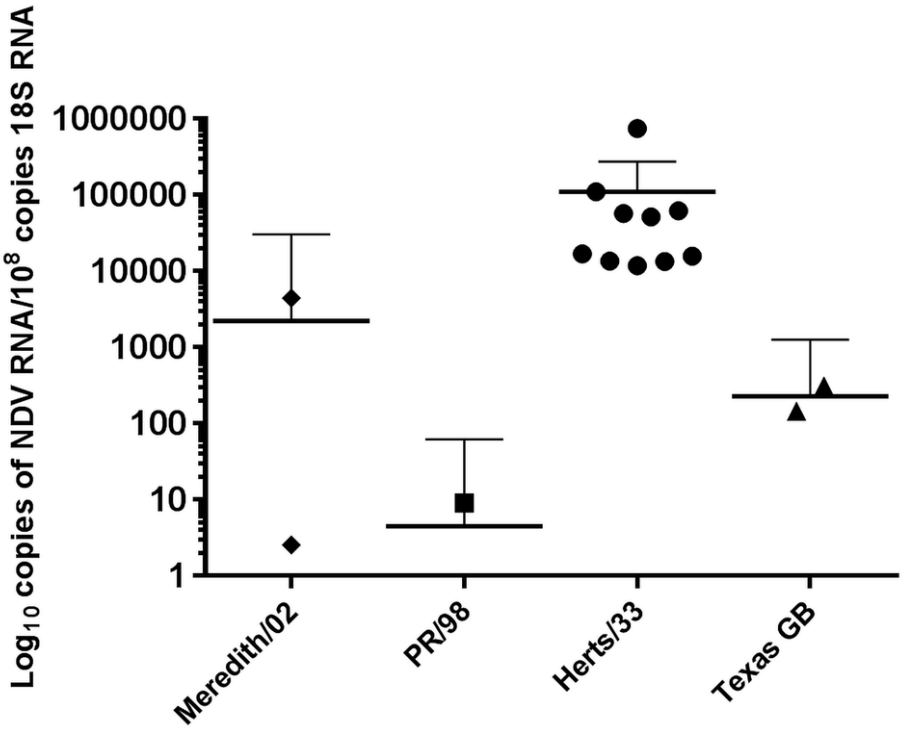


Figure 4.9 NDV RNA copy numbers in spleen samples at 2 dpc; bars represent mean values. All comparisons between viruses were significantly different ($p<0.05$, Mann-Whitney U test).

4.3.7 Virus Isolation

Virus isolation was attempted from five different tissues (brain, kidney, lung, spleen and small intestine) when PCR results were positive (Table 4.4). Virus was re-isolated from all but one PCR-positive tissue in one bird in both the Herts 33/56 and Texas GB virus groups. In both groups, the PCR-positive, virus isolation-negative sample was cerebrum.

In birds exposed to the Meredith/02 ND virus, virus was only recovered from birds euthanased at 2 and 4 dpc and not from those euthanased at 14 dpc. No virus was isolated from birds inoculated with the PR/98 virus at any time point (Table 4.4).

Animal identification	Day of euthanasia	Cerebrum	Kidney	Lung	Spleen	Duodenum
Meredith/02						
6	2	-/nd	-/nd	-/nd	+/+	-/nd
7	2	-/nd	-/nd	+/+	+/+	-/nd
1	4	-/nd	+/+	+/+	+/+	+/+
4	4	-/nd	-/nd	+/+	+/+	-/nd
2	14	-/nd	-/nd	-/nd	+/-	-/nd
3	14	-/nd	-/nd	-/nd	+/-	-/nd
5	14	-/nd	-/nd	-/nd	+/-	-/nd
8	14	-/nd	-/nd	-/nd	+/-	-/nd
9	14	-/nd	-/nd	-/nd	+/-	-/nd
10	14	-/nd	-/nd	-/nd	+/-	-/nd
Peats Ridge/98						
13	2	-/nd	-/nd	-/nd	-/nd	-/nd
19	2	-/nd	-/nd	-/nd	+/-	+/-
12	4	-/nd	-/nd	-/nd	-/nd	-/nd
16	4	-/nd	-/nd	-/nd	+/-	-/nd
11	14	-/nd	-/nd	-/nd	-/nd	-/nd
14	14	-/nd	-/nd	-/nd	-/nd	+/-
15	14	-/nd	-/nd	-/nd	-/nd	-/nd
17	14	-/nd	-/nd	-/nd	-/nd	-/nd
18	14	+/-	-/nd	-/nd	-/nd	-/nd
20	14	-/nd	-/nd	-/nd	-/nd	-/nd
Herts 33/56						
21	2	+/+	+/+	+/+	+/+	+/+
22	2	+/+	+/+	+/+	+/+	+/+
23	2	+/+	+/+	+/+	+/+	+/+
24	2	+/+	+/+	+/+	+/+	+/+
25	2	+/+	+/+	+/+	+/+	+/+
26	2	+/+	+/+	+/+	+/+	+/+
27	2	+/+	+/+	+/+	+/+	+/+
28	2	+/+	+/+	+/+	+/+	+/+
29	2	+/-	+/+	+/+	+/+	+/+
30	2	+/+	+/+	+/+	+/+	+/+
Texas GB						
12	2	-/nd	-/nd	-/nd	+/+	-/nd
20	2	-/nd	-/nd	-/nd	+/+	-/nd
11	4	+/+	+/+	+/+	+/+	+/+
16	4	+/+	+/+	+/+	+/+	+/+
18	4	+/+	+/+	+/+	+/+	+/+
19	4	+/+	+/+	+/+	+/+	+/+
13	5	+/+	+/+	+/+	+/+	+/+
14	5	+/+	+/+	+/+	+/+	+/+
15	5	+/-	+/+	+/+	+/+	+/+
17	5	+/+	+/+	+/+	+/+	+/+

Table 4.4 PCR and virus isolation in tissues (PCR / virus isolation); nd: not done.

4.3.8 Sequencing

Whole genome sequencing of the virus isolates showed that the viruses had the same fusion cleavage site sequences as previously reported.[71, 129, 131] Sequence results are shown in Table 4.5.

	F protein cleavage site motif[a]	Virulence	HN extension length
Meredith/02	^{112}**RR**Q**RR**F^{117}	Virulent	9 amino acids
PR/98	^{112}**RR**QG**R**L^{117}	Avirulent	9 amino acids
Herts 33/56	^{112}**RR**Q**RR**F^{117}	Virulent	4 amino acids
Texas GB	^{112}**RR**Q**KR**F^{117}	Virulent	6 amino acids

Table 4.5 Fusion protein cleavage site motifs and HN extension length (basic amino acids are highlighted in bold)

Partial sequencing of the F protein and HN extension from samples collected from the Meredith/02, Herts 33/56 and Texas GB inoculated birds after the infection trial were consistent with the sequence results of the virus inoculum, indicating that these regions had not mutated at these sites during replication in the birds. Due to the low virus titres in the birds infected with the PR/98 virus, sequencing was not successful on samples from these birds.

4.4 Discussion

This study investigated the pathogenicity of the atypical Australian NDV Meredith/02 by comparison with an avirulent Australian virus and two exotic velogenic viruses. The atypical Australian virus (Meredith/02), possessed a virulent F protein cleavage site sequence according to the OIE definition but did not induce severe clinical signs, which is consistent with the field observations at the time of its isolation.[9] This is unusual given that ND viruses with multiple basic amino acids at the F protein cleavage site and a phenylalanine at position 117 are usually associated with a virulent phenotype.[129]

Whilst birds exposed to the Meredith/02 virus appeared very mildly depressed for a short period of time on 3 dpc, they all completely recovered within hours. The PR/98 virus was typical for most avirulent ND viruses, in that no clinical signs were observed in inoculated birds. As expected, the velogenic viruses Herts 33/56 and Texas GB viruses induced severe clinical signs, leading to the euthanasia of all birds by 2 and 5 dpc respectively. The clinical signs seen in the birds inoculated with Herts 33/56 and Texas GB viruses were

typical for viscerotropic and neurotropic viruses, respectively and were similar to those previously reported.[101, 162]

This work has confirmed the observation made by Susta et al., that some Australian isolates of NDV obtained from 1998 – 2002 are less pathogenic in an experimental setting than other viruses with similar virulent cleavage site motifs.[104] This is despite the Meredith/02 virus and the APMV-1/chicken/Australia/9809-19-1107/1998 viruses having the same virulent F protein cleavage site of ^{12}RRQRRF^{117} and high ICPIs of 1.61 and 1.88 respectively. The Meredith/02 virus in particular appears even less pathogenic than the APMV-1/chicken/Australia/9809-19-1107/1998 virus. However previously, the Australian NDV isolates responsible for the 1998-2002 outbreaks, including the Meredith/02 virus had been described as velogens, based on F protein cleavage site sequence data and ICPI values.[56, 104] Clearly, the cleavage site sequence and ICPI data alone is not sufficient to classify pathotype, although currently these are the best measures available to predict the behaviour of an NDV.

Phylogenetic analysis of Australian ND viruses detected during the 1998-2002 outbreaks placed them within class II, genotype I, of which the majority of isolates are avirulent and found in wild birds.[81] The presence of a 9 amino acid extension to the HN protein identifies these viruses as uniquely Australian.[50, 71] The Meredith/02 isolate was shown to cluster with the other 1998-2002 isolates, although it was slightly more divergent from others in the group.[71] This slight increase in phylogenetic distance may account for the difference in pathogenicity between the 9809-19-1107 virus reported by Susta et al. and the Meredith/02 virus reported here. Additionally, Susta et al. used slightly younger 4-week old chickens, which may have led to increased susceptibility to disease.

The presence of a virulent F protein cleavage site enables the ND virus to be cleaved by proteases that are widespread throughout numerous cell types. This allows the virus to enter and replicate in a greater range of cells and organ systems than those with an avirulent cleavage site motif.[113, 163] Therefore, in accordance with its cleavage site motif, it would be expected that the Meredith/02 virus would also have a wide antigen distribution. The distribution and concentration of virus as measured by immunohistochemistry, PCR and virus isolation indicated that whilst the virus was able to replicate systemically in the spleen of all birds and in the kidney, lung and duodenum of 3/10 birds, live virus was cleared from all tissues by 14 dpc. Compared with the virulent exotic viruses, the virus load of the Meredith/02 virus was markedly reduced, corresponding with fewer and less

severe histopathological lesions, and minimal clinical signs in the inoculated birds. The antigen was primarily restricted to the mucosa at the sites of inoculation. In comparison, the viscerotropic Herts 33/56 virus preferentially targeted lymphoid tissue early in the course of infection. This indicates that the presence of a virulent F protein cleavage site in the Meredith/02 virus whilst allowing for some systemic viral replication, was not associated with the high viral loads in tissues associated with typical velogenic viruses.

In general, the pathogenicity of a virus is determined by a number of factors including the route of exposure to the virus, the immune status and immune response of the birds, and the ability of the virus to replicate and disseminate throughout the host tissues.[21] Increased virus replication and a greater inflammatory response within lymphoid tissues has been associated with increased pathogenicity of velogenic ND viruses.[164] The results of our study indicate that the low pathogenicity of the Meredith/02 virus must be attributed to regions of the viral genome other than the F protein cleavage site, given that the cleavage site contains a virulent motif. Studies using reverse genetics have also indicated that regions of the NDV genome other than the F protein cleavage site may also contribute significantly to pathogenicity.[3, 12, 120] For example, the viral replication complex has been associated with the decreased pathogenicity of the pigeon paramyxovirus for chickens, despite its virulent cleavage site motif.[12]

Overall, this experimental work has confirmed the field observations, that despite containing a virulent F protein cleavage site as defined by the OIE, the Meredith/02 isolate of NDV is not highly pathogenic for chickens. Clinical signs, pathological lesions and antigen distribution in the experimentally infected birds were instead consistent with a lentogenic or mesogenic virus.

The risk posed by these Australian viruses is unclear. This evolution of an avirulent to virulent virus is unusual and has only been described once before in Ireland.[165] Whilst the PR/98 virus has been shown to only require two nucleotide changes to become pathogenic, it is uncertain whether the Meredith/02 virus is also a significant risk to the poultry industry. Whilst the Meredith/02 virus displayed minimal pathogenicity in the field and very limited pathogenicity in an experimental setting, there is still a possibility that this virus may increase in pathogenicity given appropriate circumstances. It is possible that the Meredith/02 was not yet well adapted to chickens. High pathogenicity may evolve with intensive production systems, such as most commercial chicken operations in Australia,

due to the large numbers of birds held in such facilities allowing numerous passages of the virus. In addition, concurrent infection with agents such infectious bronchitis virus or infectious bursal disease virus may induce immunosuppression and allow for virus evolution. Immune pressure induced by vaccination may also contribute to an increased NDV mutation rate and result in increased pathogenicity. However, the genetic changes necessary for this transformation are unknown. Conversely, this virus may be a stable genetic type that may not continue evolving greater pathogenicity, and therefore have a low risk for commercial poultry. Currently, we are not able to assess this on the genetic information that we have, but knowledge of the impacts of potential mutations would be important for risk assessment and outbreak response. At the time of its detection, a conservative approach was taken and the affected flocks were depopulated. However, this approach may not be necessary if it can be demonstrated that such viruses have little risk of further evolution towards higher pathogenicity.

4.5 Conclusions

The Australian NDV, Meredith/02, whilst containing a virulent F protein cleavage site (RRQRRF) consistent with a virulent virus, was only mildly pathogenic in chickens in an experimental setting. Viral replication primarily occurred in sites of inoculation and there were no mortalities associated with the viral infection. The decreased pathogenicity of the virus may therefore be associated with regions of the genome other than the F protein cleavage site.

CHAPTER 5
VIRUS CHARACTERISATION

5.1 Introduction

Some Australian Newcastle disease viruses (NDVs) are unusual in that whilst they are classified as virulent according to the molecular sequence at the fusion protein cleavage site, they do not induce severe disease when inoculated into chickens. This was demonstrated in the previous chapter (Chapter 4: An Australian Newcastle disease virus with a virulent fusion protein cleavage site produces minimal pathogenicity in chickens). The four NDVs used in this work were Peats Ridge/98, Meredith/02, Texas GB and Herts 33/56. The Peats Ridge/98 and Meredith/02 viruses are Australian viruses, with avirulent and virulent fusion protein cleavage sites respectively. Despite containing a virulent cleavage site sequence motif, the Meredith/02 virus does not behave like a typical virulent NDV in either an experimental setting or in the field. Therefore, it was hypothesized that there may be genetic elements other than the fusion protein cleavage site in this virus that could contribute to pathogenicity.

NDV belongs to the family *Paramyxoviridae* in the genus *Avulavirus*. They comprise a negative sense, single strand RNA genome of six genes, from 3' to 5': nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and large polymerase (L). The genes encode seven (and putatively eight) proteins with the additional V and W proteins produced via RNA editing of the P gene.

In order to examine which sections of the viral genome should be targeted for further investigation into their role in virulence, the four viruses were characterised by standard virological techniques and whole genome sequence analysis. Replication in cells and embryonated eggs was compared and the amino acid sequences associated with key functional areas of each of the virus proteins were analysed, including cleavage and glycosylation sites.

The overall aim of these analyses was to determine whether there were any specific differences in the genome of the Meredith/02 virus when compared with the Herts 33/56 and Texas GB viruses, that may account for its lower than expected pathogenicity.

5.2 Materials and Methods

5.2.1 Viruses

As per Chapter 4, the four viruses used in this work were Peats Ridge/98 (Australia), Meredith/02 (Australia), Texas GB (velogenic neurotropic) and Herts 33/56 (velogenic viscerotropic). Before use, these viruses were all propagated via chorioallantoic inoculation in 10 day old embryonated SPF chicken eggs for 5 days.

5.2.2 Virus titration and mean death time in eggs

Each virus was grown in both cell culture and eggs to determine end-point titres and the mean death time in eggs (MDT). Virus titration in cell culture was conducted in DF-1 cells as per Chapter 3.2, using DMEM with incubation at 37ºC with 5% CO_2. Prior to the addition of virus, TPCK-treated trypsin (Worthington, New Jersey) was added to the media to give a final concentration of 2 µg/ml.

Viruses were also titrated in specific pathogen free eggs as per Chapter 3.3. A ten-fold dilution series was used to inoculate 100 µl of virus per egg. Eggs were then incubated for 5 days and allantoic fluid tested for the presence of virus by haemagglutination.

The mean death time (MDT) in eggs was determined for each virus as per Chapter 3.7. The MDT is the mean time for all the embryos inoculated with the most dilute virus inoculum to be killed. Briefly, two groups of five, 9-11 day old embryonated SPF eggs were inoculated with each of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} dilutions of virus, eight hours apart. Eggs were observed over 7 days and the time of egg deaths recorded. The mean death time for each virus was then calculated.

5.2.3 Whole genome sequencing

Whole genome sequencing was conducted using the MiSeq platform according to Chapter 3.12. Prior to sequencing, viruses were purified using a discontinuous sucrose gradient and ultracentrifugation. RNA was then extracted using the QIAamp Viral RNA Mini Kit (Qiagen) and reverse transcribed using the Superscript III transcriptase kit (Invitrogen). Double strand cDNA was then synthesized and amplified using random PCR with the Expand High Fidelity kit (Roche) and the 454 amplification primer (see Appendix 1, Table A2 for the primer sequence). PCR products were then purified using the Wizard® SV Gel

and PCR Clean-Up System (Promega) and sequenced using the MiSeq (Illumina). The genomes were assembled using read mapping and *de novo* assembly with CLC Genomics Workbench (CLC Genomics Workbench v.6.0.5.).

5.2.4 Sequence analysis

Sequence alignments and phylogenetic analysis were undertaken using Geneious (Geneious R8, v. 8.0.5) with the PhyML plugin. Sequences for comparison were primarily based on Dimitrov et al. and represent viruses from all NDV classes and genotypes.[82] Nucleotide alignments were conducted using Geneious with default settings of a 65% cost matrix, gap open penalty of 12, gap extension penalty of 3 and refinement iterations of 2. The most appropriate model for phylogenetic tree construction was determined using the jModelTest2.[166] A maximum likelihood tree was then created using the general time-reversible model with invariant sites (0.420), a gamma distribution (2.595) and 1000 bootstrapped replicates using Geneious. The number of replicates was chosen based on the number of sequences examined (n=23).[167]

Amino acid sequences were aligned and examined for known pathogenicity determinants and glycosylation sites. Alignments were conducted using Geneious global alignment using default settings of Blosum 62 cost matrix, gap open penalty of 9 and gap extension penalty of 3. N-linked glycosylation sites were predicted using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). BLAST searches were performed to compare amino acid sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

5.2.5 Embryo histopathology and immunohistochemistry

After the initial growth of virus stocks in 10-day-old embryonated chicken eggs, the embryos and chorioallantoic membranes were harvested after 5 days and fixed in 10 per cent neutral buffered formalin for 24-48 hrs. The membranes and multiple sections of the whole embryos were then processed for histology and stained with haematoxylin and eosin. The Q91-6 NDV NP monoclonal antibody was used for immunohistochemistry. Further histology methodology can be found in Chapter 3.4.

5.3 Results

5.3.1 Virus titration

The virus titres for each of the virus isolates in both DF-1 cells and in SPF eggs can be seen in Table 5.1.

Virus	DF-1 cells	Eggs (SPF)
Meredith/02	$10^{5.75}$ $TCID_{50}$/ml	$10^{9.3}$ EID_{50}/ml
PR/98	$10^{5.75}$ $TCID_{50}$/ml	$10^{10.0}$ EID_{50}/ml
Herts 33/56	$10^{7.50}$ $TCID_{50}$/ml	$10^{9.2}$ EID_{50}/ml
Texas GB	$10^{7.75}$ $TCID_{50}$/ml	$10^{9.5}$ EID_{50}/ml

Table 5.1 Virus titres in cell culture and SPF eggs.

The cytopathic effect of each of the viruses in DF-1 cells was typical for paramyxoviruses, with fusion of cells and the formation of syncytia. The cytopathic effect for the Texas GB virus can be seen in Figure 5.1.

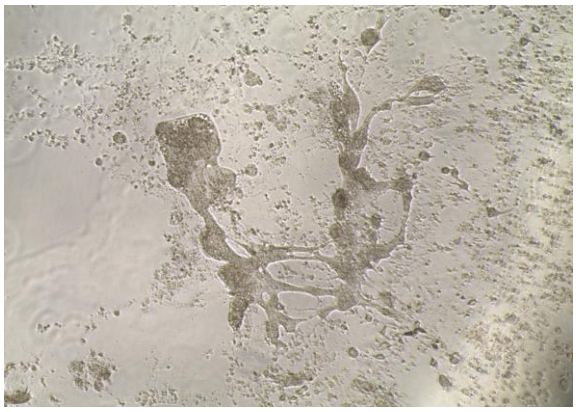


Figure 5.1 The cytopathic effect induced by Texas GB in DF-1 cells, consisting of syncytial cells.

5.3.2 Mean death time in eggs

The MDT and corresponding classification for each of the viruses can be seen below in Table 5.2. The classification of the MDT is as follows, lentogenic: >90 hours, mesogenic 60 – 90 hours, velogenic <60 hours.

Virus	Mean Death Time (hours)	Pathotype
Meredith/02	68	Mesogenic
PR/98	116	Lentogenic
Herts 33/56	44	Velogenic
Texas GB	60	Velogenic

Table 5.2 Mean death time in eggs. Lentogenic, >90 hours, mesogenic 60-90 hours, velogenic <60 hours.

As expected, the PR/98 virus was classified as lentogenic and the Herts 33/56 and Texas GB viruses were velogenic. The Meredith/02 virus was classified as mesogenic.

5.3.3 Sequence analysis

After translation and alignment, the sequences were compared with previously published NDV sequences and important functional regions of genomes analysed. Results of these analyses are shown below in Figures 5.2 – 5.7.

The Meredith/02 sequence showed 99.6% nucleotide agreement with the previously published Meredith/02 sequence in Genbank (AY935490). Similarly, there was 99.8% similarity between the Peats Ridge/98 sequence obtained here and the Peats Ridge/98 Genbank sequence (AY935491). The Texas GB virus showed between 98.3-100% nt similarity with two partial Texas GB sequences in Genbank (AY935490, JN872191). The Herts 33/56 virus was most similar overall to the Ulster/67 virus in Genbank with 90% nt similarity (AY562991.1). However when BLASTn was used against Herts ’33 virus sequences from Czegledi, et al., it had 100% nt identity to partial fusion protein sequences from Herts 33/56 viruses (AY1701401.1, AY170138.1).[168]

The full-length sequences of all four viruses were 15,186 nucleotides in length. Results from alignments of both nucleotides and amino acids of individual genes and proteins can be seen in Tables 5.3 – 5.8. Overall, the most variability was found within the phosphoprotein gene and the highest conservation within the polymerase gene.

N gene	Meredith/02	PR/98	Herts 33/56	Texas GB
Meredith/02		99.591	95.092	95.919
PR/98	98.980		95.501	96.124
Herts 33/56	90.646	90.918		93.670
Texas GB	89.490	90.102	87.959	

Table 5.3 N gene distance matrix. Distances are represented as percentage similarities. Nucleotide similarities are represented with grey shading and amino acid similarities are unshaded.

P gene	Meredith/02	PR/98	Herts 33/56	Texas GB
Meredith/02		98.228	86.352	88.872
PR/98	99.074		87.618	89.632
Herts 33/56	87.500	88.089		86.364
Texas GB	88.763	89.184	87.205	

Table 5.4 P gene distance matrix. Distances are represented as percentage similarities. Nucleotide similarities are represented with grey shading and amino acid similarities are unshaded.

M gene	Meredith/02	PR/98	Herts 33/56	Texas GB
Meredith/02		99.176	92.595	93.407
PR/98	99.178		93.419	93.956
Herts 33/56	88.082	88.721		91.771
Texas GB	88.676	89.498	87.352	

Table 5.5 M gene distance matrix. Distances are represented as percentage similarities. Nucleotide similarities are represented with grey shading and amino acid similarities are unshaded.

F gene	Meredith/02	PR/98	Herts 33/56	Texas GB
Meredith/02		98.915	92.948	92.043
PR/98	98.857		92.405	92.405
Herts 33/56	88.989	89.350		89.873
Texas GB	89.651	89.892	87.665	

Table 5.6 F gene distance matrix. Distances are represented as percentage similarities. Nucleotide similarities are represented with grey shading and amino acid similarities are unshaded.

HN gene	Meredith/02	PR/98	Herts 33/56	Texas GB
Meredith/02		98.135	90.878	91.387
PR/98	98.967		90.909	91.246
Herts 33/56	88.290	88.754		89.617
Texas GB	88.809	88.924	87.406	

Table 5.7 HN gene distance matrix. Distances are represented as percentage similarities. Nucleotide similarities are represented with grey shading and amino acid similarities are unshaded.

L gene	Meredith/02	PR/98	Herts 33/56	Texas GB
Meredith/02		99.503	95.650	96.508
PR/98	99.116		96.011	96.733
Herts 33/56	90.529	90.907		94.877
Texas GB	90.242	90.695	90.083	

Table 5.8 L gene distance matrix. Distances are represented as percentage similarities. Nucleotide similarities are represented with grey shading and amino acid similarities are unshaded.

5.3.3.1 N protein

The nucleocapsid gene encodes a protein of 489 amino acids with most of the variability seen in the carboxyl terminus. However, even in this region there was good sequence similarity between Texas GB and the Australian viruses. The amino acid alignment of the N protein for all four viruses is shown in Figure 5.2.

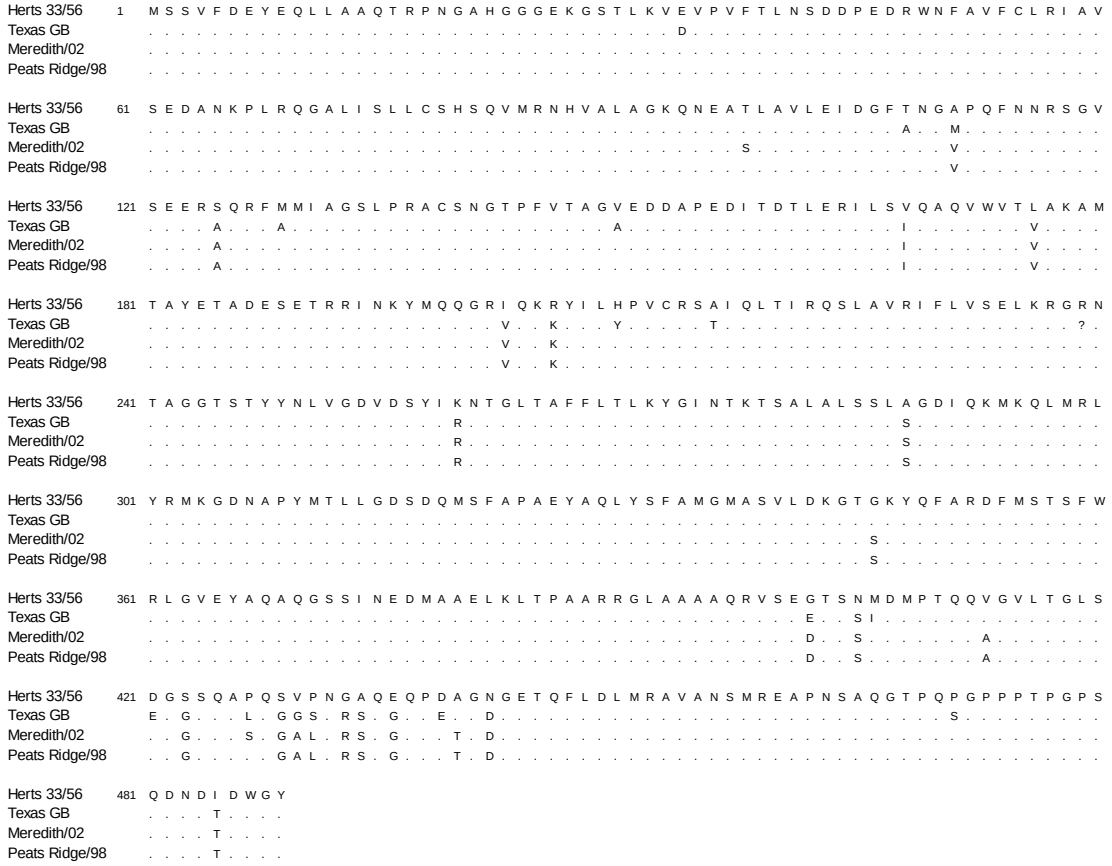


Figure 5.2 N protein alignment

5.3.3.2 P protein

The phosphoprotein gene encodes a 395 amino acid protein and is the most variable of all the proteins. The phosphoprotein gene also contains an RNA editing site which allows for the production of a secondary V protein via the insertion of G nucleotide and a subsequent frame-shift. The amino acid alignment of the P protein for all four viruses is shown in Figure 5.3. There are a number of sequence differences at various sites, with differences unique to the Australian viruses present at 22 positions, predominantly in the 3' half of the gene. The significance of these changes is currently unknown.

Figure 5.3 P protein alignment

5.3.3.3 V protein

As previously discussed, the V protein is produced by RNA editing of the P gene and is 239 aa in length. The Australian viruses ranged between 84.94% - 87.40% aa in similarity to the Herts 33/56 and Texas GB sequences, particularly throughout the cysteine rich region (highlighted in blue in Figure 5.4).

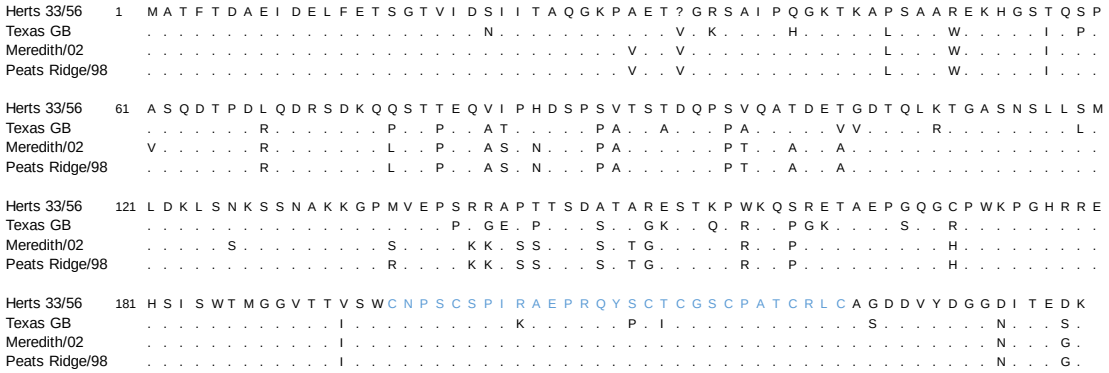


Figure 5.4 V protein alignment. The cysteine rich region is highlighted in blue.

5.3.3.4 M protein

The matrix protein is 364 aa in length. It initially localises to the nucleus during virus replication and transcription, whilst in later stages it is associated with budding of the NDV virus from the cellular membrane.[169] To enable nuclear localisation, it contains a nuclear localisation signal (highlighted in blue in Figure 5.5).[170] The signal contains two clusters of basic amino acids between positions 246 and 263. There was a non-basic amino acid substitution R259G in the Meredith/02 virus. Otherwise, there was good sequence conservation between all four viruses.

Figure 5.5 M protein alignment. The nuclear localisation signal is highlighted in blue.

5.3.3.5 F protein

The NDV fusion protein (563 aa) contains the cleavage site, which is the main determinant of pathogenicity and is highlighted in red in Figure 5.6. The Peats Ridge/98 virus contained the avirulent cleavage site motif of ^{112}RRQGRL^{117}, whilst the other three viruses had virulent cleavage sites with the Meredith/02 and Herts 33/56 cleavage sites being identical, ^{112}RRQRRF^{117}. The fusion protein contains 6 potential N-linked glycosylation sites (highlighted in blue). All of these sites were identical throughout the fours viruses except for the first site, in which the Herts 33/56 virus contained a K. Other important structural components, the two heptad repeat domains are boxed.[171]

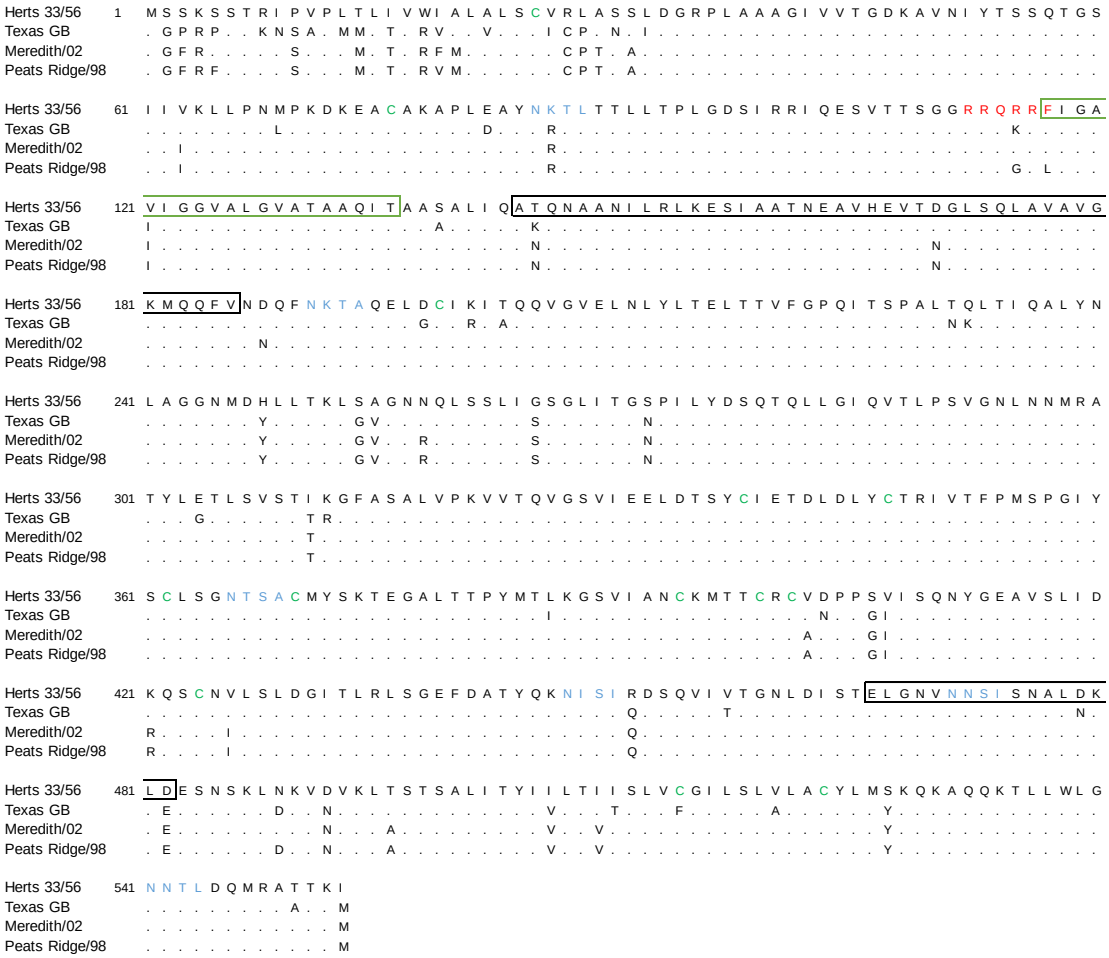


Figure 5.6 F protein alignment. The cleavage site is indicated in red, glycosylation sites in blue, cysteine residues in green, the fusion peptide in a green box and heptad repeats in black boxes.

5.3.3.6 HN protein

The HN protein, like the F protein also has 6 potential N-linked glycosylation sites, however only four of these are functional and are highlighted in blue in Figure 5.7. [172]. The Australian viruses contained a different motif (NSSG) at the first HN glycosylation site compared with the exotic viruses. Heptad repeats (HR1 and HR2) were conserved in all viruses and are seen between residues 73-88 for HR1 and 96-116 for HR2.[173] The HN length varies between the viruses as a result of extensions of 3, 6, 9 and 9 aa for the Herts 33/56, Texas GB, Meredith/02 and PR/98 viruses respectively.

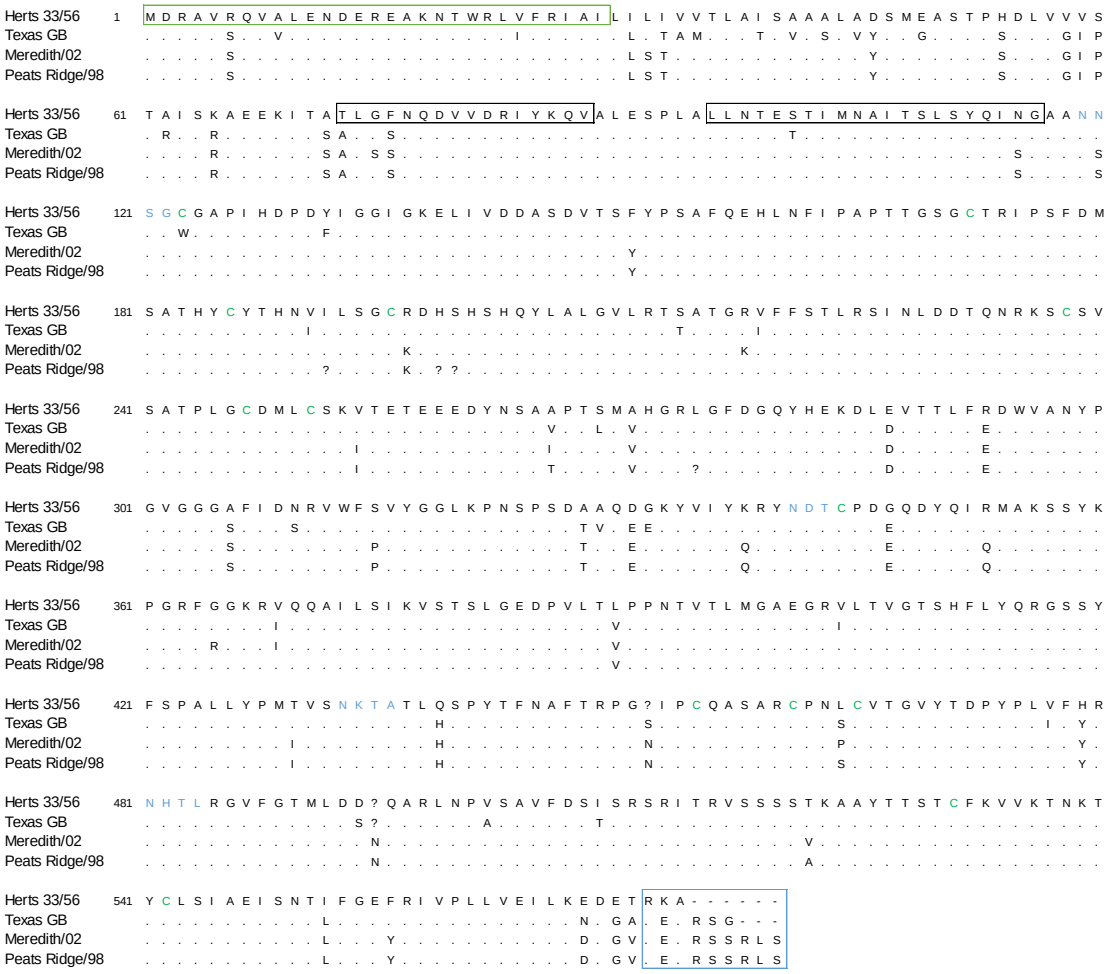
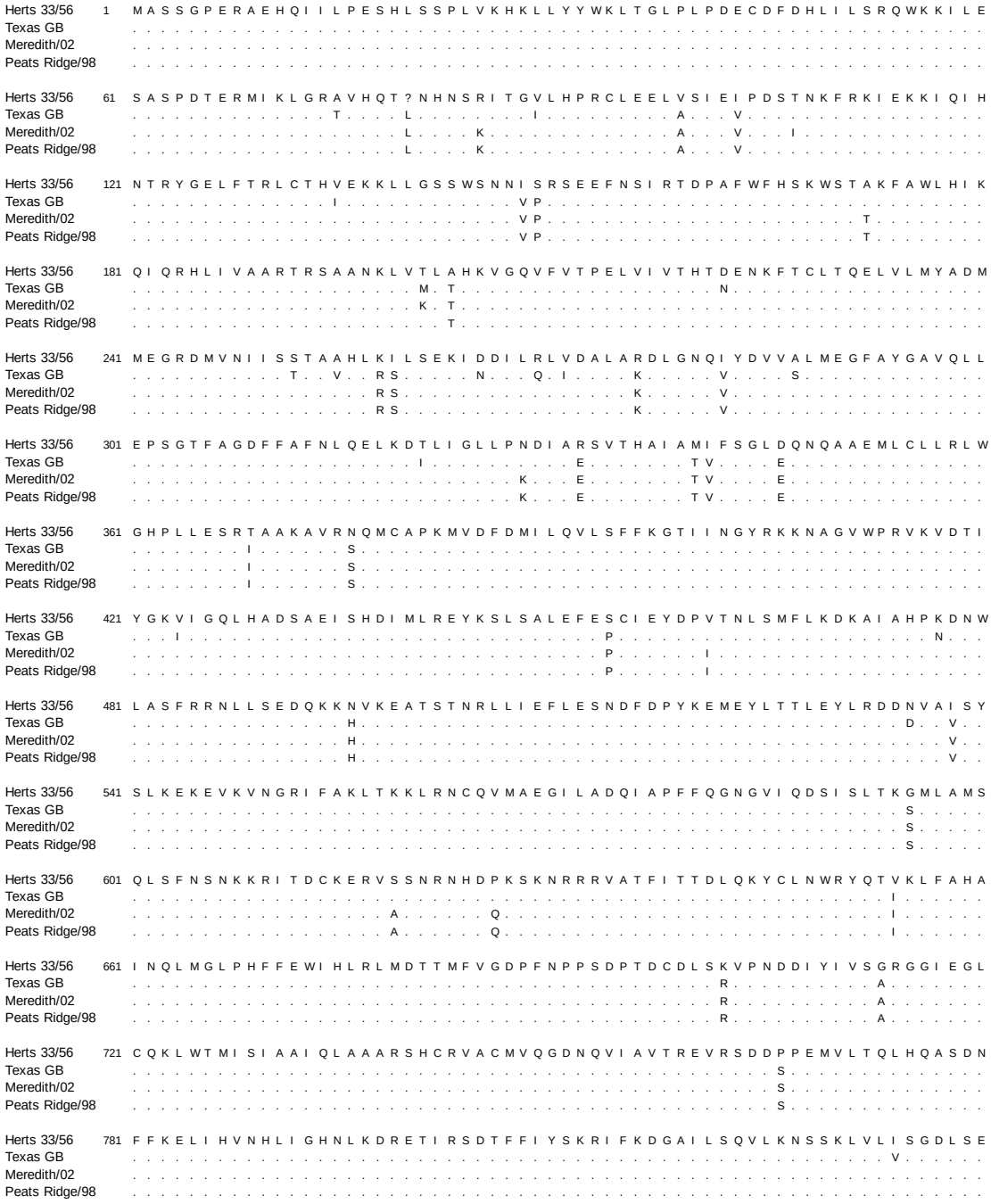
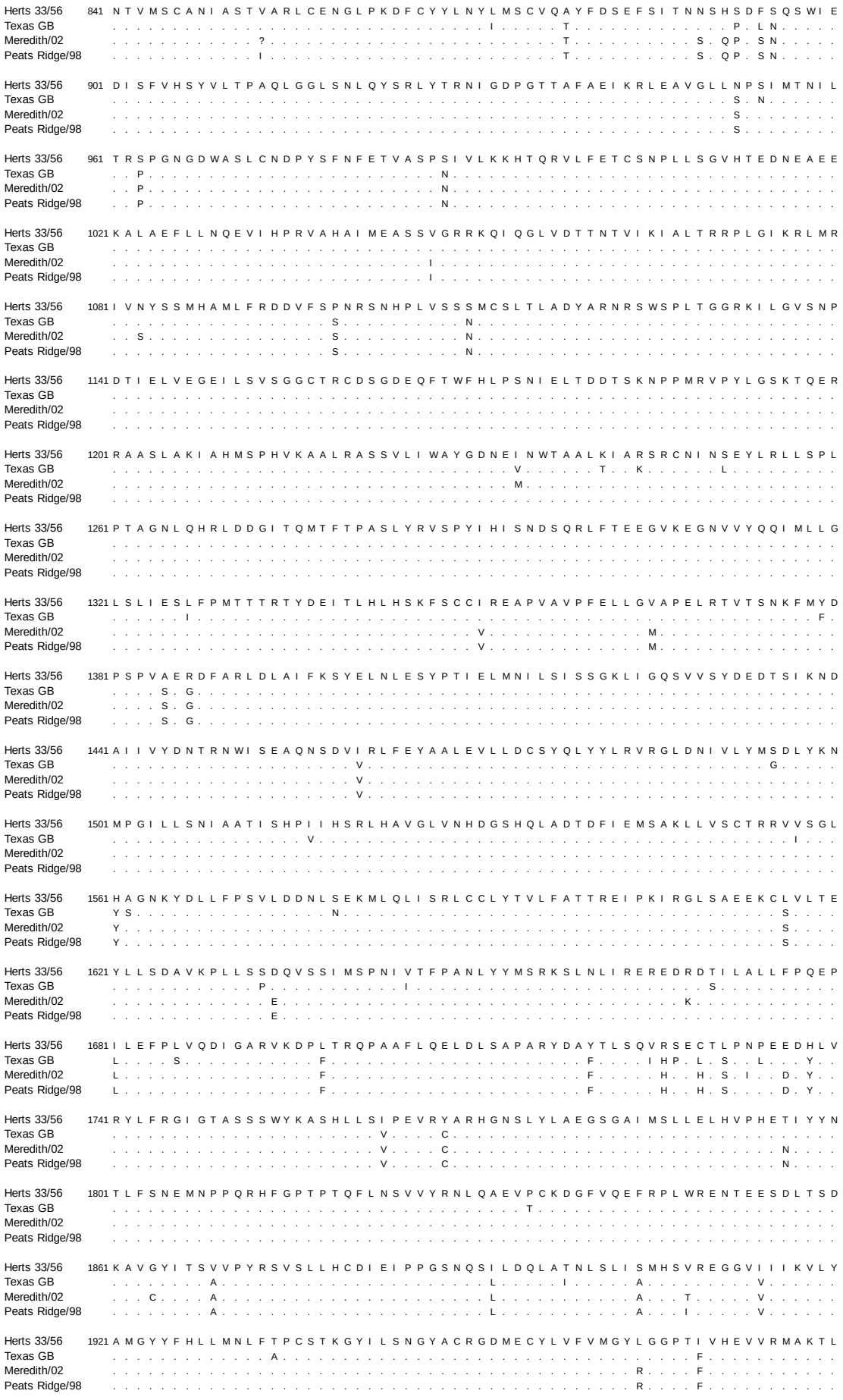


Figure 5.7 HN protein alignment. Glycosylation sites are indicated in blue, cysteine residues in green, the transmembrane peptide in a green box, heptad repeats in black boxes and the HN extension in a blue box.

5.3.3.7 L protein

The L gene encodes for the longest NDV protein, the large polymerase protein, which is 2204 amino acids in length. As seen in Figure 5.8, there was generally good sequence conservation across the protein alignment, particularly between the Texas GB and Australian viruses.




Herts 33/56 841 N T V M S C A N I A S T V A R L C E N G L P K D F C Y Y L N Y L M S C V Q A Y F D S E F S I T N N S H S D F S Q S W I E
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 901 D I S F V H S Y V L T P A Q L G G L S N L Q Y S R L Y T R N I G D P G T T A F A E I K R L E A V G L L N P S I M T N I L
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 961 T R S P G N G D W A S L C N D P Y S F N F E T V A S P S I V L K K H T Q R V L F E T C S N P L L S G V H T E D N E A E E
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1021 K A L A E F L L N Q E V I H P R V A H A I M E A S S V G R R K Q I Q G L V D T T N T V I K I A L T R R P L G I K R L M R
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1081 I V N Y S S M H A M L F R D D V F S P N R S N H P L V S S S M C S L T L A D Y A R N R S W S P L T G G R K I L G V S N P
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1141 D T I E L V E G E I L S V S G G C T R C D S G D E Q F T W F H L P S N I E L T D D T S K N P P M R V P Y L G S K T Q E R
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1201 R A A S L A K I A H M S P H V K A A L R A S S V L I W A Y G D N E I N W T A A L K I A R S R C N I N S E Y L R L L S P L
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1261 P T A G N L Q H R L D D G I T Q M T F T P A S L Y R V S P Y I H I S N D S Q R L F T E E G V K E G N V V Y Q Q I M L L G
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1321 L S L I E S L F P M T T T R T Y D E I T L H L H S K F S C C I R E A P V A V P F E L L G V A P E L R T V T S N K F M Y D
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1381 P S P V A E R D F A R L D L A I F K S Y E L N L E S Y P T I E L M N I L S I S S G K L I G Q S V V S Y D E D T S I K N D
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1441 A I I V Y D N T R N W I S E A Q N S D V I R L F E Y A A L E V L L D C S Y Q L Y Y L R V R G L D N I V L Y M S D L Y K N
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1501 M P G I L L S N I A A T I S H P I I H S R L H A V G L V N H D G S H Q L A D T D F I E M S A K L L V S C T R R V V S G L
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1561 H A G N K Y D L L F P S V L D D N L S E K M L Q L I S R L C C L Y T V L F A T T R E I P K I R G L S A E E K C L V L T E
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1621 Y L L S D A V K P L L S S D Q V S S I M S P N I V T F P A N L Y Y M S R K S L N L I R E R E D R D T I L A L L F P Q E P
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1681 I L E F P L V Q D I G A R V K D P L T R Q P A A F L Q E L D L S A P A R Y D A Y T L S Q V R S E C T L P N P E E D H L V
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1741 R Y L F R G I G T A S S S W Y K A S H L L S I P E V R Y A R H G N S L Y L A E G S G A I M S L L E L H V P H E T I Y Y N
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1801 T L F S N E M N P P Q R H F G P T P T Q F L N S V V Y R N L Q A E V P C K D G F V Q E F R P L W R E N T E E S D L T S D
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1861 K A V G Y I T S V V P Y R S V S L L H C D I E I P P G S N Q S I L D Q L A T N L S L I S M H S V R E G G V I I I K V L Y
Texas GB
Meredith/02
Peats Ridge/98
Herts 33/56 1921 A M G Y Y F H L L M N L F T P C S T K G Y I L S N G Y A C R G D M E C Y L V F V M G Y L G G P T I V H E V V R M A K T L
Texas GB
Meredith/02
Peats Ridge/98

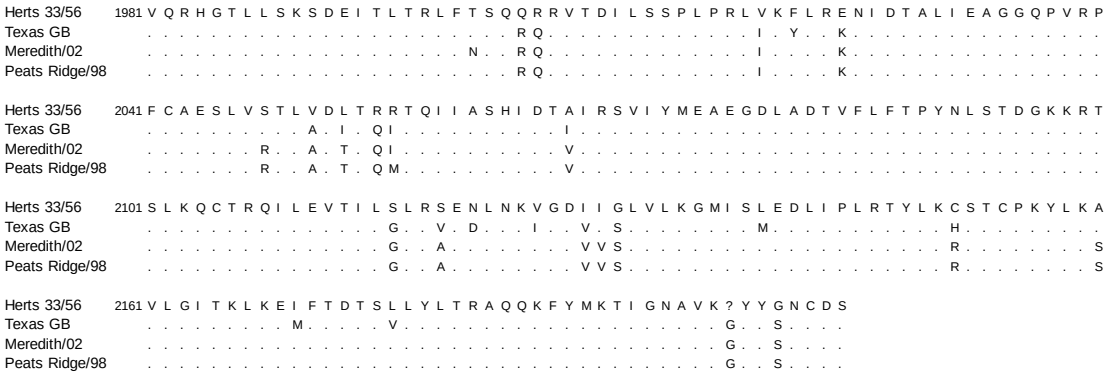


Figure 5.8 L protein alignment

5.3.3.8 Intergenic sequences

The intergenic sequences and gene boundaries for each of the genes have been aligned in Figure 5.9. The gene end and start sequences for each of the proteins were extremely well conserved, however there was significant diversity in the intergenic regions, particularly between the F-HN and HN-L proteins.

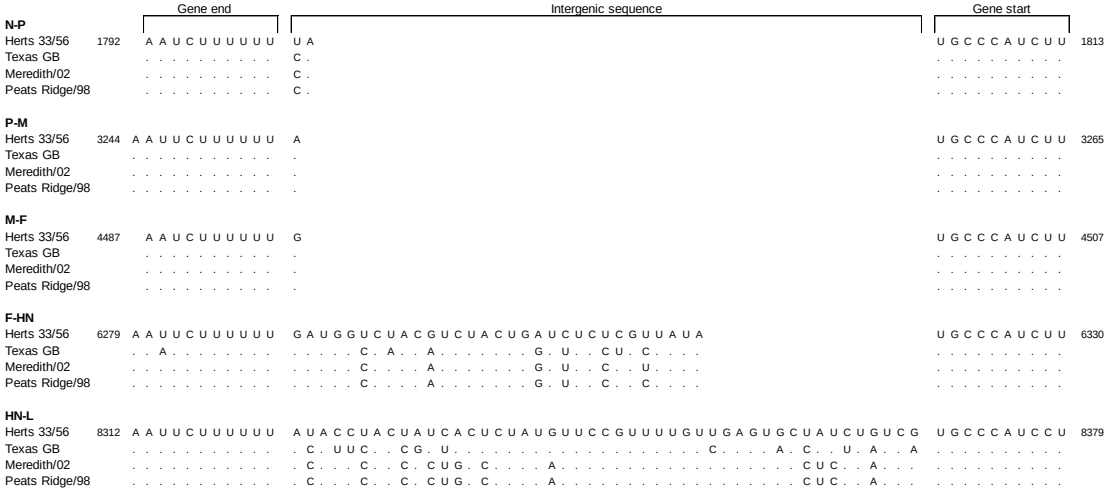


Figure 5.9 Gene boundaries and intergenic sequences

5.3.4 Phylogenetics

The phylogenetic relationship of the F gene of viruses used in this study was examined in the form of a maximum likelihood tree (Figure 5.10). As expected from previous studies, the Australian viruses cluster within Class II, genotype I.[104] The Herts 33/56 virus in a separate genotype close to genotype I and Texas GB in genotype 2.[82, 168]

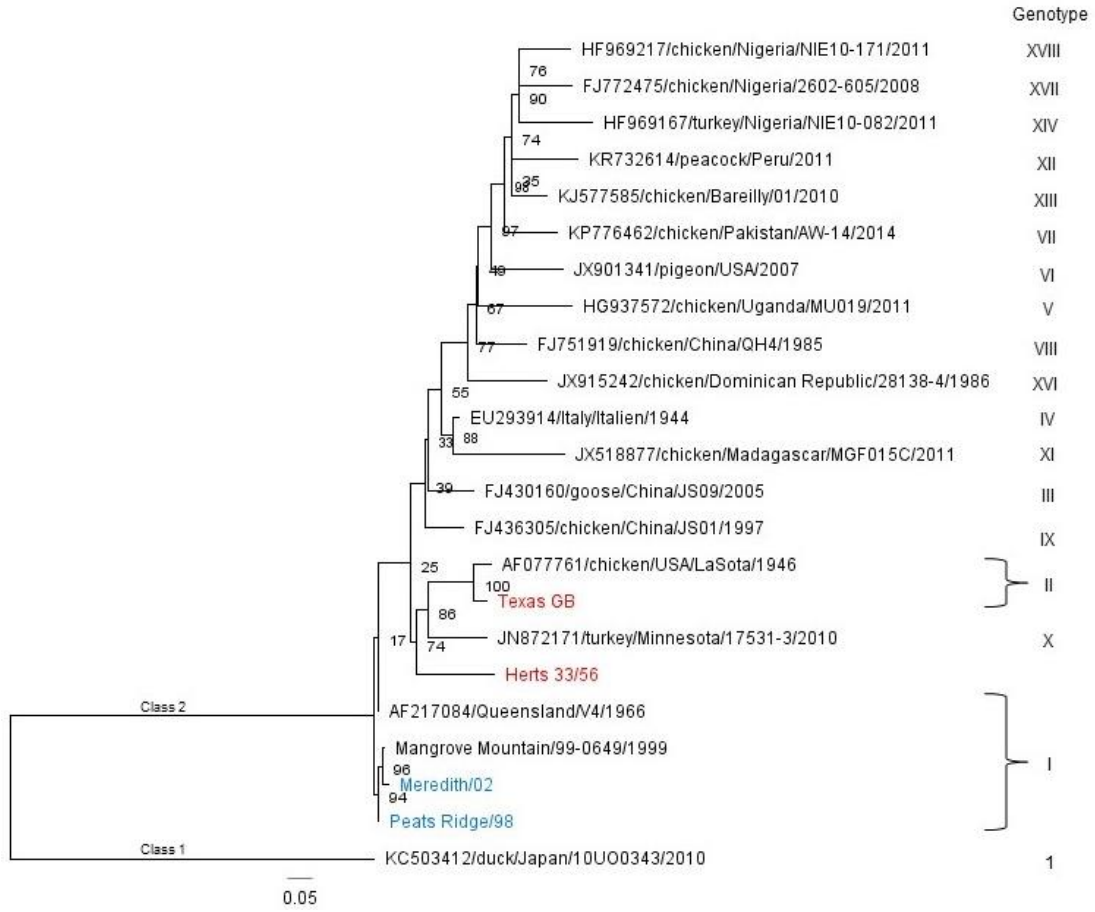


Figure 5.10 Phylogenetic tree showing the relationship between Australian viruses (blue) and velogenic viruses (red) used in this study. The maximum-likelihood phylogenetic tree based on the full length fusion gene has been estimated using a general time-reversible model with a gamma distribution and invariant sites. Bootstrap values are shown as a percentage of 1000 replicates. The virus genotype is indicated in roman numerals. The scale bar represents nucleotide substitutions per site.

5.3.5 Embryo histopathology

The chorioallantoic membranes (CAM) and embryonic tissues were examined for NDV antigen staining. The immunohistochemical staining characteristics are represented in Table 5.9. All embryos showed strong staining throughout the CAM. However the Peats Ridge/98 virus was only associated with staining on the endodermal surface as seen in

Figure 5.11A. In addition, all viruses induced staining throughout the internal organs of the embryo, apart from the Peats Ridge/98 virus, in which no staining was detected.

Tissue	Meredith/02	Peats Ridge/98	Herts 33/56	Texas GB
Chorioallantoic membrane	+++	+++	+++	+++
Internal organs[a]	+++	-	+++	+++

Table 5.9 Immunohistochemical straining of embryos infected with each of the four ND viruses using the Q91-6 monoclonal antibody [a]Internal organs include lung, liver and kidney; +++ widespread staining, - no staining.

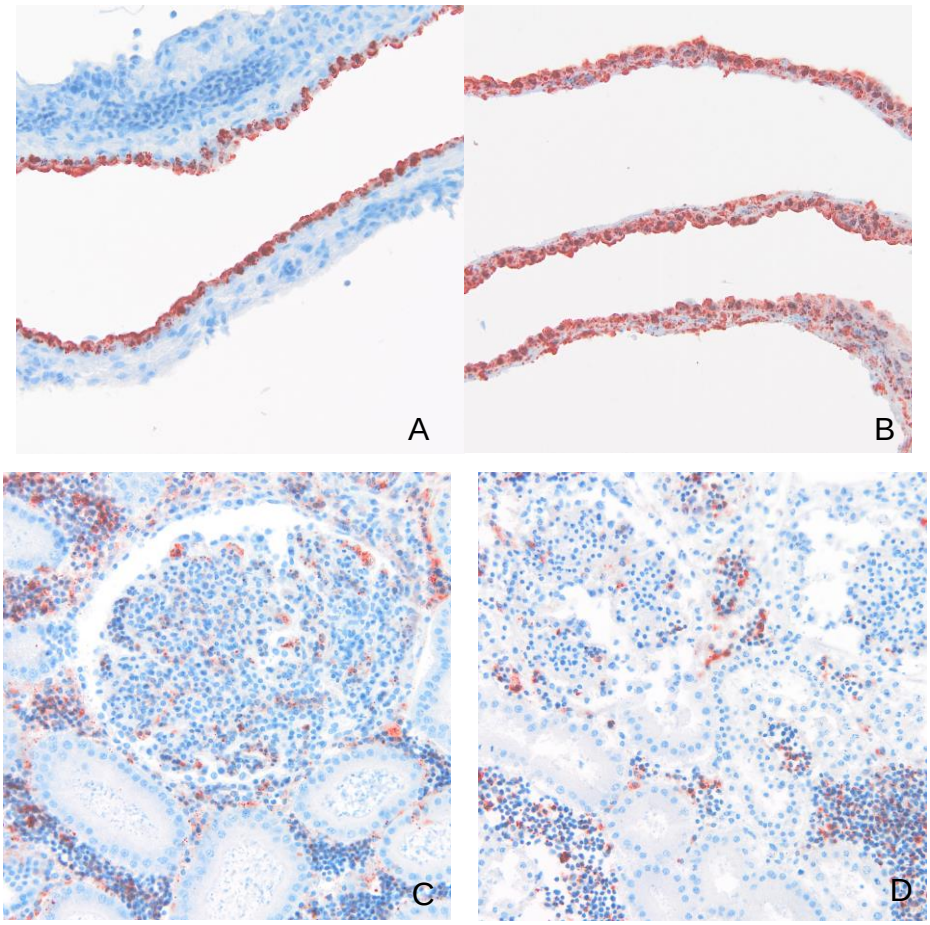


Figure 5.11 Immunohistochemistry of embryos and CAMs stained with NDV MAb Q91-6. A - Peats Ridge/98, B. Meredith/02 CAM, C. Herts 33/56 embryo kidney, D. Meredith/02 embryo kidney.

5.4 Discussion

This study has examined virological and molecular characteristics of four NDVs; two virulent viruses, Herts 33/56 and Texas GB and two Australian viruses, Meredith/02 and Peats Ridge/98. The virus of interest in this study is Meredith/02 which appears to be less pathogenic for poultry than is indicated by its virulent fusion protein cleavage site. In characterising this virus in comparison with some typical velogenic and lentogenic viruses, it was hoped to gain insight into why the Meredith/02 virus displays a less pathogenic phenotype.

The exotic virulent viruses Herts 33/56 and Texas GB grew to titres per ml of $10^{7.5}$ $TCID_{50}$ and $10^{7.75}$ $TCID_{50}$ respectively in DF-1 cells, whereas the Australia viruses, Meredith/02 and Peats Ridge/98 grew to titres of $10^{5.75}$ $TCID_{50}$ and $10^{5.5}$ $TCID_{50}$, respectively. However, when inoculated into embryonated eggs (a more sensitive and natural culture system), there was minimal difference in titres, with all viruses reaching a titre of between 10^{9} EID_{50} and $10^{10.3}$ EID_{50} per ml. The lower titres seen with the Australian viruses in cell culture may therefore be artefactual due to the sensitivity of the DF-1 cells.

The mean death time in embryonated eggs is a well-established classification method for NDV.[174] This study confirmed that the Herts 33/56 and Texas GB viruses both kill embryos rapidly, indicating a velogenic pathotype. The Peat Ridge/98 virus took an extended period of time >90 hours to kill embyros, consistent with a lentogenic pathotype. However, the Meredith/02 virus killed all ten embryos of the highest dilution in 68 hours. This classifies it as a mesogenic virus. The intracerebral pathogenicity index (ICPI) for the Meredith/02 virus has previously been calculated to be 1.61.[71] ICPI values greater than or equal to 0.7 are classified as virulent (either velogenic or mesogenic). Therefore, based on both the MDT and ICPI, it appears that the Meredith/02 virus is a mesogenic virus.

Whole genome sequencing of each of the isolates was undertaken to compare each of the viral genes and determine if there were any significant nucleotide and/or amino acid changes which may influence the pathogenicity of the viruses. Some of these viruses had previously been sequenced at the time that they were first isolated, however, all viruses were sequenced again to take advantage of recent developments in sequencing technologies. Gene boundaries, including intergenic sequences were also aligned. Gene

start and end sequences were well conserved and intergenic regions ranged from 1-47 nucleotides in length, consistent with published NDV sequences.[175]

When each of the viral proteins were aligned, the sequences contained a number of differences between the exotic and Australian strains, however none of these differences have previously been reported to be associated with pathogenicity. For example, the M protein showed a non-basic amino acid substitution at R259G, the influence of which is unknown at this stage.

Glycosylation sites were examined because they are important sites of post-translation modification that can significantly alter protein interactions.[176] The only difference noted between the viruses was in the first HN glycosylation site, in which the Australian viruses contained the motif NSSG. However, this amino acid sequence is also seen in other virulent viruses (e.g. Genbank AGL09175.1), so is unlikely to be associated with viral attenuation.[134]

The phylogenetic analysis confirmed previous classifications of these four viruses.[104, 168] The Australian class II genotype I viruses also cluster with other viruses of low pathogenicity such as the V4 vaccine strain, other avirulent Australian viruses and viruses from wild birds.[82] Whilst the original Herts 33 virus was designated a class II genotype IV virus, the related Herts 33/56 virus appears more closely related to the genotype I viruses, despite its high pathogenicity. The Texas GB virus clusters with class I genotype II viruses of which the majority are virulent, however this group also contains vaccine strains such as La Sota. Whilst viruses in genotypes I and II continue to circulate worldwide, they are distinct from those in genotype VII which are currently causing severe disease outbreaks in Asia and the Middle East.

The histopathological analysis of the embryonated eggs showed that the location of viral replication within the eggs varied with the virus strains. As expected, the Peats Ridge/98 virus, with an avirulent cleavage site was restricted to replication in the CAM on the endodermal surface of the allantoic membrane. The virus was unable to invade into the mesenchymal tissue to reach the chorionic (ectodermal) layer. This is consistent with previous descriptions of lentogenic Australian viruses.[52] All 3 other viruses, including the Meredith/02 virus were able to replicate on both the endodermal and the ectodermal surface of the membrane, as well as throughout the internal organ parenchyma of the

developing embryo. This indicates that the Meredith/02 fusion protein is able to be cleaved by furin-like proteases as with other virulent viruses. As such, the cleavability of the fusion protein of the Meredith/02 virus is unlikely to be the cause of the decreased pathogenicity associated with the virus.

Currently, there are no molecular methods available to differentiate between mesogenic and velogenic viruses and both are reportable to the OIE. However, if specific molecular signatures were found to differentiate the two, this may not be the case. Given that there a large number of amino acid variations that could account for the variation in pathogenicity between the mesogenic Meredith/02 virus and the velogenic viruses, a broad approach analysing of the roles of the individual proteins in pathogenicity may be useful. Analyses could include *in-vitro* work investigating innate immune system antagonism, virus entry and budding from cells or *in-vivo* studies using full-length clones with interchanged genes.

5.5 Conclusions

The characteristics of these four NDVs, including viral titre, mean death time, amino acid sequence, phylogeny and embryo immunohistochemistry are consistent with their respective fusion protein cleavage sites and predicted pathogenicity. The Meredith/02 virus, despite its minimal pathogenicity in chickens, contains a virulent fusion protein cleavage site and is classified as mesogenic according to the mean death time in eggs. In addition, it produces immunohistochemical staining in chicken embryos consistent with a virulent virus. However, there were no obvious sequence motifs within the Meredith/02 genome to account for the mild clinical signs observed experimentally and in the field in poultry.

CHAPTER 6
THE INNATE IMMUNE RESPONSE TO THE AUSTRALIAN MEREDITH/02 VIRUS

6.1 Introduction

Chapters 4 and 5 have examined various characteristics of an Australian Newcastle virus (NDV), Meredith/02, which has a polybasic fusion protein cleavage consistent with the OIE definition of a virulent NDV, but does not behave as a virulent virus when inoculated into chickens experimentally. It is likely that there are other molecular determinants in this virus that somehow mitigate against the virulent cleavage site sequence.

The Meredith/02 virus has been shown to have a decreased ability to replicate systemically within infected birds when compared with other velogenic viruses. This could be associated with an increased host innate immune response to the virus. For example, the virus may lead to greater production of antiviral cytokines in the early stages of infection, limiting its ability to replicate and spread. To investigate this, this chapter will compare features of the chicken innate immune response to the Meredith/02 virus and the viscerotropic velogenic Herts 33/56 virus.

The avian immune response to NDV comprises both adaptive and innate immunity. Adaptive immunity is derived from antibodies directed at the fusion and haemagglutinin proteins on the virus surface, along with cell-mediated immunity in the form of CD4+ and CD8+ T lymphocytes.[177] The innate immune system on the other hand, comprises physical barriers, phagocytes, complement, natural killer cells and cytokines. The production of certain cytokines in chickens has been shown to increase in response to infection with viruses such as avian influenza virus and NDV.[178] After initial exposure to a virus, recognition of pathogen associated molecular patterns (PAMP) results in binding of pattern recognition receptors such as Toll-like receptors (TLR).[179] Binding of TLRs induces the activation of genes encoding cytokine production including the type I interferon family comprising interferon-α (IFN-α) and interferon- β (IFN-β).[177, 180] IFN-α and IFN-β, once released, bind to class II cytokine receptors, IFNAR1 and IFNAR2 respectively (Figure 6.1). This activates a signaling cascade via the Janus kinase (Jak) and signal transducer and activation of transcription (STAT) pathway, which results in the activation

of IFN-stimulated genes (ISG) which have a wide range of effects, including further cytokine production, cell degranulation and adenosine monophosphate production.[181] One of these ISGs is the myxovirus resistance protein (Mx). Mx is an IFN-induced GTPase, which has been shown to have antiviral activity in mice and humans using a number of mechanisms, including inhibiting early viral replication.[182] The role of the Mx protein is more controversial in the chicken with some studies indicating an important role for Mx in the antiviral response to influenza and others showing that Mx has limited antiviral activity.[180, 183, 184] However, whilst the definitive role that Mx plays in the defense against ND is not clear, it has been evaluated in this study to further compare the type I IFN pathways induced against NDV isolates of differing pathogenicity.

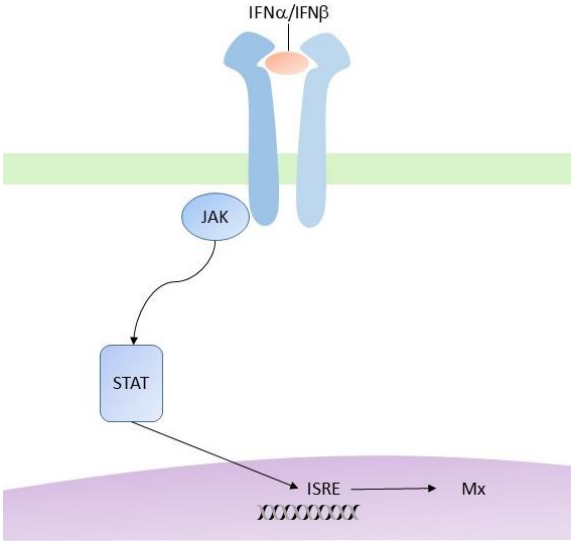


Figure 6.1 The interferon signaling pathway. ISRE, interferon-stimulated response element. Diagram adapted from.[182]

In the case of NDV *in vivo*, it has been shown that the cytokine response to viral infection, in particular type I IFN induction (IFN-α/β), varies with the virulence of the virus. More virulent viruses such as CA02 have been shown to induce greater production of IFN-α, IFN-γ, IL-1β and IL-6 compared with lentogenic viruses such as La Sota *in vivo.*[185] However, the role of cytokines in pathological damage to the host has not yet been elucidated. Certainly some cytokines eg. interleukin-2 appear to be beneficial in clearing NDV by reducing viral titres.[186] In the case of highly pathogenic avian influenza virus infection in humans, the increased production of cytokines, in the form of a 'cytokine storm', has been shown to be deleterious to the infected person.[187] However, the 'cytokine storm' has not been demonstrated in the context of NDV in chickens as yet.

The NDV V protein, formed during RNA editing of the P gene, has been found to antagonize interferon production by targeting phosphorylated STAT1 and may also play a role in viral pathogenesis.[11, 150] Studies using reverse genetics have also identified the viral replication complex (N, P and L proteins) as influencing the pathogenicity of the virus. Therefore, in order to further understand molecular basis for the decreased pathogenicity of the Australian Meredith/02 virus, the roles of the P and V proteins were examined in terms of their effect on innate immunity.

Initially, this study compared the ability of each virus to induce expression of IFN-α, IFN-β and Mx in cell culture. Then, the ability of the P and V proteins of the Meredith/02 virus and Herts 33/56 virus to antagonize IFN-α, IFN-β and Mx was assessed. It was hypothesized that the Meredith/02 virus, being of decreased pathogenicity would not be able to suppress the innate immune pathways to the same extent as the Herts 33/56 virus, leading to comparatively greater cytokine levels in cell culture at early time points post infection.

6.2 Materials and Methods

6.2.1 Infection of DF-1 cells

DF-1 cells were grown as described in Chapter 3.2.1 and used to seed 24-well tissue culture plates. Four separate plates were used to enable cells to be harvested at four different time points. When cells were 80% confluent, they were infected with either the Herts 33/56 virus or Meredith/02 virus at an MOI of 1 or Poly I:C (10 µg) using DMEM growth media with 5% foetal calf serum. Cells were infected in triplicate with three control wells per plate.

Cells were harvested at four time points, 0, 6, 12 and 24 hours by removing the overlying media and adding 140 µl MagMAX^{TM} Lysis/Binding solution directly to each well as per Chapter 3.8. Lysed cells were then stored at -80^{o}C until RNA isolation, which was performed according to Chapter 3.8 using the Kingfisher Flex extraction machine.

6.2.2 Gene expression SYBR Green Reverse Transcriptase PCR

The RNA isolated in Chapter 6.2.1 was used to quantify the expression of IFN-α, IFN-β and Mx using previously published primers.[188-190] The sequences of these primers can be

found in Appendix 1, Table A4. The SYBR Green RNA to C_T kit (Applied Biosystems) was used for PCR as per Chapter 3.9.2. The RNA for each gene was quantified using 28S rRNA as an endogenous control for normalisation. All data were then analysed using the comparative C_T method with the control cells at time 0 hr as the reference sample.

6.2.3 Phosphoprotein gene cloning

The P genes from both the Herts 33/56 and Meredith/02 viruses were cloned by ligation into the pCAGGS expression vector.[191] Primers were designed to incorporate restriction enzyme sites at either end and a haemagglutinin (HA) epitope tag at the 5' end of the primer to allow for easy identification of the generated protein by Western blot if required. The structure of the primers (blue arrows) can be seen in Figure 6.2.

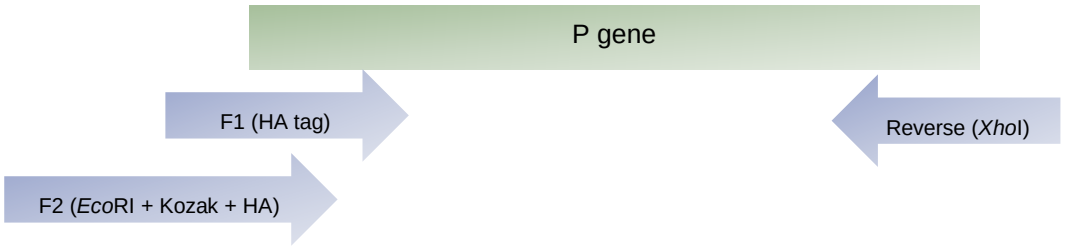


Figure 6.2 Primer design for P gene cloning.

Two overlapping forward primers were required to span the distance from the 5' end of the F2 primer to the start of the P gene and one reverse primer was used to incorporate the *Xho*I restriction site. The first forward primer (F1) contained the HA tag and the start of the P gene, whilst the second forward primer (F2) contained the *Eco*RI restriction site and the Kozak sequence, overlapping the F1 primer at the HA tag. The exact primer sequences can be found in Appendix 1, Table A3.

RNA for cloning was extracted from allantoic fluid that had been inoculated with the Herts 33/56 and Meredith/02 viruses. The QIAamp Viral RNA Mini kit (Qiagen) was used to isolate the RNA as per Chapter 3.8.

PCR reactions were conducted using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). Reactions were conducted in 50 µl volumes using 25 µl 2X reaction mix, 10 µl template RNA, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2 µl SuperScript III RT/ Platinum Taq Mix and 11 µl nuclease-free water. The F1 forward primer was used in the first reaction with the following cycling conditions:

cDNA synthesis: 50ºC for 30 minutes x 1 cycle
Denaturation: 94ºC for 2 minutes x 1 cycle
PCR amplification: 94ºC for 15 seconds (denaturation), 55ºC for 30 seconds (annealing), 68ºC for 1 minute (extension) x 40 cycles
Final extension: 68ºC for 5 minutes x 1 cycle
4ºC ∞

The resulting PCR product was then used as the template in the next PCR reaction using the second forward primer (F2). The Q5 high fidelity 2X master mix (New England Biosciences) was used for the second PCR with the following reagents in a 25 µl volume: 12.5 µl 2X Q5 master mix, 1.25 µl F2 primer (10 µM), 1.25 µl reverse primer (10 µM), 1 µl of the template cDNA and 9 µl of nuclease-free water. The following cycling conditions were used:
Denaturation: 98ºC for 30 seconds x 1 cycle
PCR amplification: 98ºC for 8 seconds (denaturation), 72ºC for 68 seconds (annealing and extension) x 35 cycles
Final extension: 72ºC for 2 minutes x 1 cycle
4ºC ∞

A 1% agarose gel was used to load all 25 µl of the final PCR product with an expected band size of 1.1 kb. When the correct band was obtained it was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) as described in Chapter 3.13.2. The cDNA was then quantified using the NanoDrop.

The pCAGGS vector and the P gene cDNA from both the Herts 33 and Meredith/02 viruses were digested with the restriction enzymes *Eco*RI and *Xho*I to produce sticky ends and to ensure directional cloning. The reactions were conducted in 50 µl volumes as follows: 5 µl 10X H buffer (Promega), 5 µl 10X Acetylated BSA, 1 µl *Eco*RI enzyme, 1 µl *Xho*1 enzyme, 31 µl nuclease free water and 7 µl cDNA. The amount of cDNA used was between 1-3 µg. The tubes were incubated for 3 hours at 37ºC.
Gel purification was used to ensure that only digested and linearised DNA was used in subsequent steps. A 1% agarose gel was used to load approximately 40 µl of the digested cDNA. If the correct sized bands were obtained, the gel was excised and cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega).

The P genes of both the Herts/33 and Meredith/02 viruses were ligated into pCAGGS vectors with a control vector for comparison. The ligation reactions were conducted in 10 µl volumes using 1 µl 10X T4 DNA ligase buffer (Promega), 1 µl pCAGGS vector DNA, 1 µl insert DNA (P gene) and 1 µl T4 DNA ligase (Promega). In the case of the control vector, 7 µl of nuclease free water was added in place of the insert DNA. The reactions were incubated overnight at 4°C.

Transformation of the ligated plasmids was achieved in Top 10 F *Escherichia coli* cells and LB agar plates with ampicillin added at 100 mg/ml, according to Chapter 3.13.5.

A colony PCR was undertaken to ensure that the correct insert was present in the bacterial colony as per Chapter 3.13.6. Colonies with the correct insert were purified (Chapter 3.13.7) and sequenced using Sanger sequencing with the pCAGGS forward primer (Chapter 3.11).

6.2.4 V protein

The NDV V protein is formed after RNA editing of the P gene. An additional G nucleotide is inserted at the P gene editing site, resulting in a +1 frameshift. In order to insert the extra G nucleotide, two rounds of PCR were required using overlapping primers (Appendix 1, Table A3). Initially, the F1 and R1 primers were used to insert the additional C nucleotide on the complementary strand. Then the F2 and R2 primers were used to insert the G nucleotide on the template strand. The products of these two PCRs were mixed and then a PCR reaction using the F1 and R2 primers resulted in the final product. The reactions can be seen in Figure 6.3. Separate F2 and R1 primers were designed for the Herts 33/56 and Meredith/02 viruses due to sequence differences around the editing site.

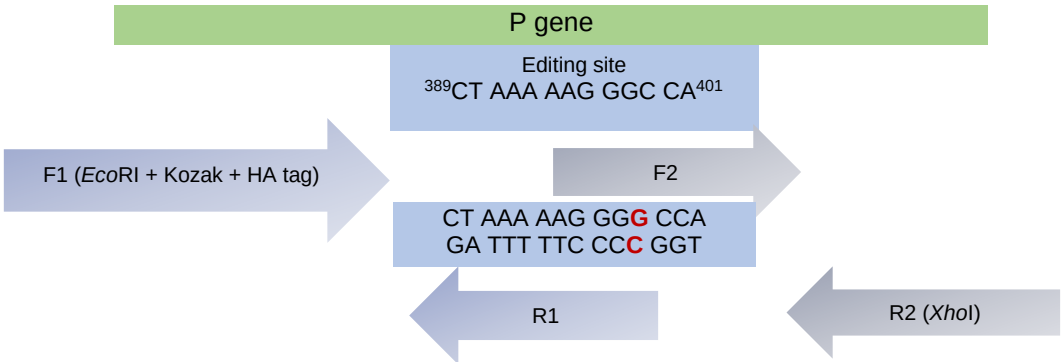


Figure 6.3 Primer design for V gene cloning

The first rounds of PCR reactions (F1/R1 and F2/R2) were undertaken using the Q5 high fidelity 2X master mix as in Chapter 6.2.1, however the annealing temperature was increased from 55^{o}C to 60^{o}C. The P gene cDNA with the attached HA tag and EcoRI restriction site was used as the template.

The second round of PCR (F1 and R2) was also conducted with the Q5 high fidelity 2X master mix: 12.5 µl 2X Q5 master mix, 1.25 µl F2 primer (10 µM), 1.25 µl reverse primer (10 µM), 1 µl template (F1/R1 product), 1 µl template (F2/R2 product) and 8 µl of nuclease-free water. The reaction conditions are as above (Chapter 6.2.1).

The final product was gel purified (Chapter 3.13.3) and digested with *Eco*RI and *Xho*I. The digested products were then ligated into the pCAGGS vector as in Chapter 6.2.1. Following this, the vectors were transformed, purified and sequenced in the same manner as for the P gene clones (Chapter 6.2.3).

6.2.3 Transfection

DF-1 cells were grown as described in Chapter 3.2.1 and used to seed two, 24-well tissue culture plates. When cells were 80% confluent, they were transfected with either the pCAGGS-Herts-P, pCAGGS-Herts-V, pCAGGS-Meredith-P, pCAGGS-Meredith-V or pCAGGS-GFP plasmids according to Chapter 3.14.1. The pCAGGS-GFP plasmid had previously been constructed in the Marsh laboratory (Glenn Marsh, pers. comm). Each of the plasmids were transfected into 6 individual wells with 6 control wells left untransfected. After 24 hours, half of the wells transfected with each of the plasmids were also transfected with Poly I:C to mimic viral infection. At 48 hours, cells were examined and transfection efficiency in the pCAGGS-GFP transfected wells calculated by counting the number of fluorescent cells per four 10X fields. This transfection efficiency was then used to estimate the ability of the other P and V gene plasmids to transfect cells. Finally, media was removed and RNA isolated as per Chapter 3.8.

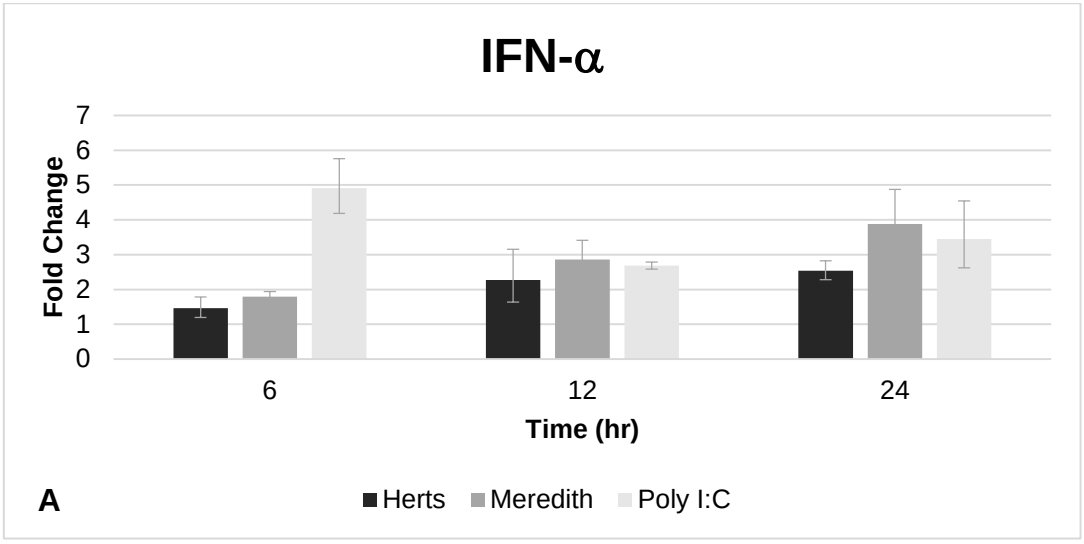
6.2.4 PCR amplification of interferon genes

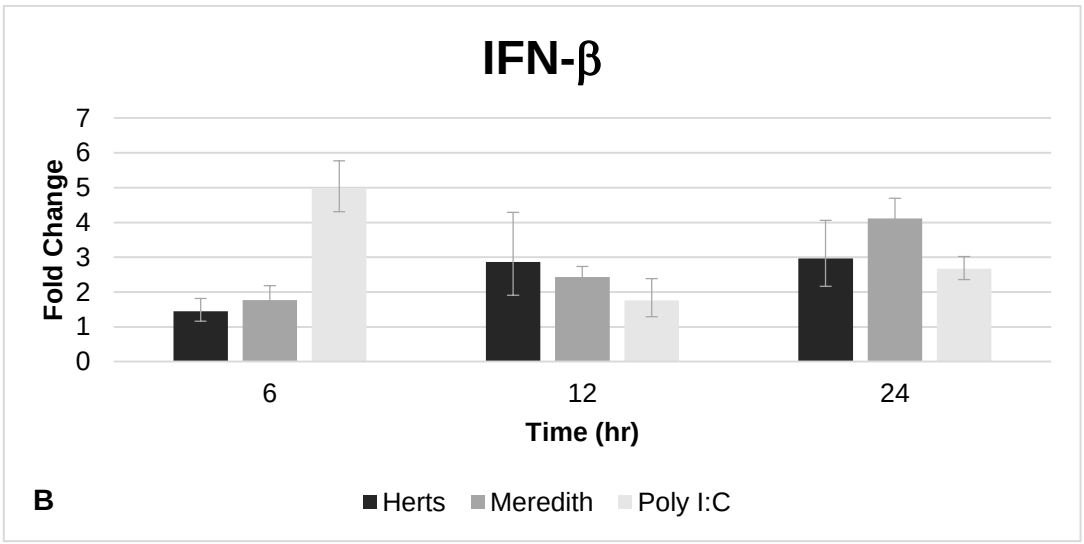
RNA from Chapter 6.2.3 was used in SYBR green RT-PCR reactions to quantify the levels of IFN-α, IFN-β and Mx mRNA. The PCR method is outlined in Chapter 3.9.2 and primer sequences can be found in Appendix 1, Table A4. The expression of each gene in DF-1 cells when stimulated with Poly I:C was compared with the respective unstimulated cells and expressed as a fold change.

6.3 Results

6.3.1 Interferon expression

The expression of type I interferon genes, IFN-α, IFN-β and Mx in DF-1 cells after infection with NDV Herts 33/56, Meredith/02 or Poly I:C at 6, 12 and 24 hours post-infection is shown in Figure 6.4. Results have been normalized to 28S rRNA are expressed as fold changes from control cells at 0 hours. The columns reflect mean fold changes from three biological replicates and error bars reflect the 95% confidence intervals.





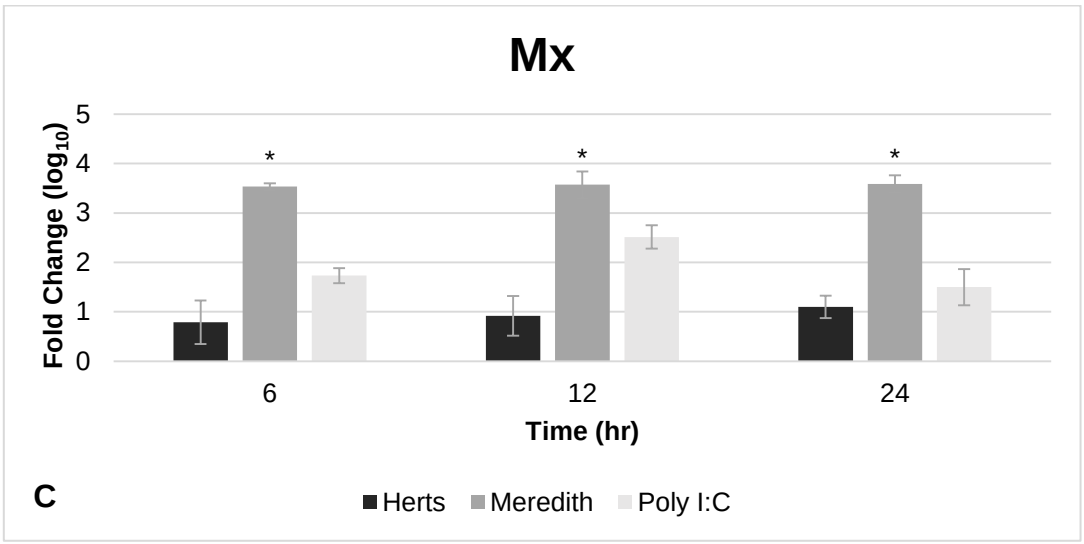


*Figure 6.4 IFN-α (A), IFN-β (B) and Mx (C) mRNA expression in DF-1 cells after infection with NDV Herts 33/56, Meredith/02 or Poly I:C. Results are expressed as mean fold changes of three biological replicates with error bars representing 95% confidence intervals. *Comparisons between Herts 33/56 and Meredith/02 p<0.05.*

After stimulation with Poly I:C to mimic viral infection, as expected, the DF-1 cells exhibited a 5 fold increase in both IFN-α and IFN-β mRNA. This fold change decreased slightly at 12 hours and there was a minimal increase again at 24 hours. When infected with Herts 33/56 virus, IFN-α expression levels increased over time from 1.5-fold at 6 hours to 2.5-fold at 24 hours. Similarly, IFN-β levels increased from a fold change of 1.5 at 6 hours to 3 at 24 hours. Interferon expression levels also increased over time after infection with the Meredith/02 virus. Fold changes were slightly higher with the Meredith/02 virus, from 1.8 at 6 hours to 3.9 at 24 hours with IFN-α and from 1.8 at 6 hours to 4.1 at 24 hours with IFN-β. However, when comparing the fold changes for each virus at each time point, the differences were not significant (Student's T-test, $p<0.05$).

The Mx protein mRNA was also found to increase in response to both viral infection and Poly I:C stimulation. Fold changes were much greater than those seen with the interferons and results have been expressed using a log scale. Overall, the expression of Mx mRNA in response to Meredith/02 virus infection was much greater than that seen with Herts 33/56 virus infection at each time point. For example, at 6 hours, the Herts 33/56 virus induced an 6-fold change, whereas the Meredith/02 virus induced a 3,415-fold change. The fold changes for Mx mRNA expression were compared between the Herts 33/56 and Meredith/02 viruses and the differences at all three time pointswere all found to be significant (Student's T-test, $p<0.05$).

6.3.2 Interferon antagonism

As seen in Chapter 6.3.1, the stimulation of cells with Poly I:C is able to induce expression of both IFN-α and IFN-β mRNA, although the fold-changes were not large. The V protein of NDV is known to antagonize interferon production and so the effect of both the P and V proteins of the Herts 33/56 and Meredith/02 ND viruses on interferon mRNA expression was investigated. The P and V proteins from both viruses were transfected into DF-1 cells, followed by Poly I:C stimulation and quantification of IFN-α and IFN-β mRNA expression.

Cells transfected were also transfected with the pCAGGS-GFP construct and were examined for fluorescence at 48 hours to assess transfection efficiency (Figure 6.5). Transfection efficiency at this time was approximately 90% and the cells were approximately 95% confluent.

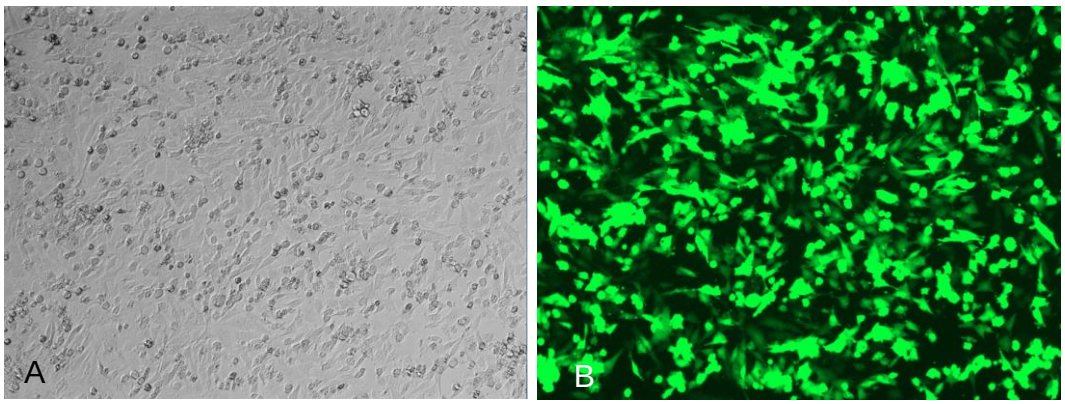


Figure 6.5 DF-1 cells transfected with pCAGGS-GFP and examined by light microscopy (A) and fluorescent microscopy (B)

SYBR green RT-PCR was used to quantify IFN-α and IFN-β and the results of these experiments can be seen in Figure 6.6. The error bars reflect the 95% confidence intervals around the mean from three biological replicates.

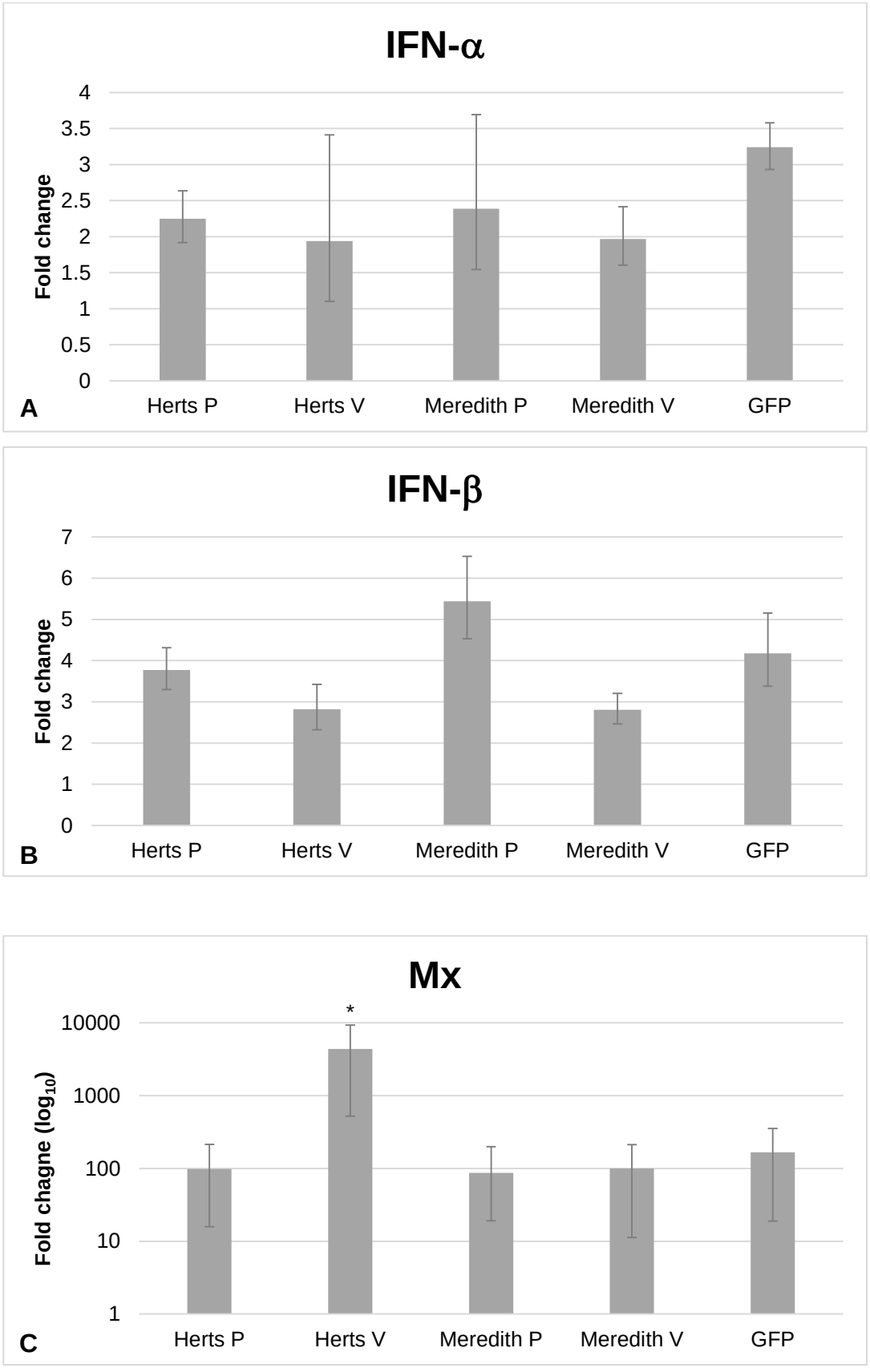


*Figure 6.6 Expression of IFN-α (A), IFN-β (B) and Mx (C) mRNA after transfection with pCAGGS-Herts-P/V or pCAGGS-Meredith-P/V and stimulation with Poly I:C. Results are normalized to 28S rRNA and expressed as the mean fold change from the transfected but unstimulated control cells. Error bars represent 95% confidence intervals. * statistically significant results, p<0.05.*

Stimulation with Poly I:C resulted in a positive fold change of both IFN-α and IFN-β mRNA as seen in the last column in Figure 6.8 A and B respectively, where the cells were only transfected with the pCAGGS-GFP plasmid prior to stimulation. When the cells were transfected with the pCAGGS-P plasmid, the fold change for IFN-α was slightly less than that seen with the pCAGGS-GFP plasmid for both the Herts 33/56 and Meredith/02 viruses. For IFN-β, the fold change for the Herts 33/56 P gene was only slightly decreased from the pCAGGS-GFP level, whereas for the Meredith/02 virus the fold change was increased. For both viruses, when the cells were transfected with the pCAGGS-V genes, the fold change of both IFN-α and IFN-β was less than that of the pCAGGS-GFP gene and less than the respective pCAGGS-P gene, although none of these differences were statistically significant.

When the effect of transfection of each gene on Mx mRNA expression was investigated, Poly I:C stimulation was seen to increase Mx expression in all cases. Compared with pCAGGS-GFP transfection, the Herts 33/56 P gene, Meredith/02 P gene and Meredith/02 V gene all slightly decreased Mx expression. However, when the Herts 33/56 V gene was transfected, the fold change in Mx mRNA expression was seen to increase significantly compared with all other plasmids.

6.4 Discussion

This study has investigated components of the chicken innate immune system, specifically the interferon pathway, to attempt to understand why there are differences in pathogenicity between the Australian Meredith/02 NDV and a typical velogenic viscerotropic ND virus, Herts 33/56. The innate immune system provides the initial response to viral infection before the adaptive immune system has time to develop. If a virus is able to antagonize the innate immune system early in the course of infection, it may be able to replicate sufficiently so that the adaptive immune system is also rendered less effective.

Given that the Meredith/02 virus behaves less pathogenically than the Herts 33/56 virus, it was hypothesized that the Meredith/02 virus may not antagonize interferon to the same extent. This means that the innate immune response to Meredith/02 would be comparatively greater, resulting in decreased virus replication and limited systemic spread. This would then lead to a greater period of time to allow the adaptive immune response to develop and enhance the antiviral effect. It therefore follows that the innate immune

response to velogenic viruses such as Herts 33/56 might be decreased, inducing less interferon production and allowing the virus to replicate to a greater degree early in the course of infection.

Initially, the type I interferon response was investigated by infecting DF-1 cells with the two viruses and Poly I:C and comparing IFN-α, IFN-β and Mx mRNA expression levels by real-time PCR over 24 hours. The Poly I:C is a synthetic construct which mimics double-stranded RNA and stimulates the immune system in a similar way to a viral infection and in this work can be thought of as a control. The 24 hour time period, using an MOI of 1 was chosen as it would allow for enough virus replication to occur to induce a cytokine response without inducing a cytopathic effect. As expected, the levels of both IFN-α and IFN-β increased over the 24 hours with both viruses and Poly I:C. The magnitude of the type I interferon expression (as indicated by size of the fold change relative to control cells), was fairly modest with a greatest fold change of 5 seen for IFN- β at 6 hrs after Poly I:C stimulation. However, this minimal change is consistent with other work using Poly I:C in DF-1 cells [192] and with NDV [193]. The expression pattern of the two interferons was similar at each time point which was also expected, given that the interferons are of the same cytokine class (IFN type I). This pattern of expression gives further validation to the results.

When comparing the two viruses, the expression of type I interferons over 24 hours showed that whilst the Australian Meredith/02 virus induced a slightly greater mean cytokine response than that produced by the Herts 33/56 virus, there was no statistically significant difference between the two viruses. However, this slightly higher mean interferon response induced by Meredith/02 was consistent with our hypothesis that the less pathogenic virus should induce a greater interferon response.

One of the more interesting findings from the work, involved the expression of the Mx protein mRNA. The Meredith/02 virus induced a strong production of Mx mRNA, which was consistent at all time points. On the other hand, Herts 33/56 was only able to induce a slight increase in Mx mRNA production, which was less than that seen with Poly I:C. The Mx protein is induced by type I interferons and therefore, the gene expression pattern would be expected to follow that seen with IFN-α and IFN-β. However, it is possible that the small increase in type I interferon produced by Meredith/02, whilst not being statistically significant, was able to greatly up-regulate the transcription of the Mx gene.

Again, the results are consistent with the hypothesis that the less pathogenic virus (Meredith/02) would induce the production of more antiviral proteins early in the course of infection.

There are limited studies that have examined the innate immune system response to NDV. Previous studies have used different virus strains, cell types, time frames and cytokines to analyse cytokine expression compared with the current study, which makes it difficult to appraise these results. There are however, a few studies in which parallels can be drawn with this study. In an experiment conducted by by Ecco et al., the cytokine response was analysed when birds were infected *in-vivo*. It was shown that cytokines IFN-γ, IFN- β and IL-6 were increased in splenic sections from birds infected with typical viscerotropic velogenic viruses (particularly at 3 dpi), when compared with an Australian virulent ND virus and a mesogenic virus.[193] There was only a minimal increase in fold change for IL-2 and IFN-β with fold-changes typically less than 5, as seen with IFN- β in our work. Another study using chicken splenocytes showed that induction of type I and II interferons was greater with virulent NDV compared with a lentogenic strain at 6 hr.[185] Both of these studies contrast with the *in-vitro* results of the current study.

Other studies comparing the cytokine responses to avian influenza viruses of differing pathogenicity have produced variable results One study showed that highly pathogenic viruses (H5N1) produce a weaker type I IFN response than the less pathogenic H3N2 virus, which is consistent with our hypothesis.[194] However, in another study comparing the cytokine response in the lung tissue of chickens infected with LPAI or HPAI, at 24 hrs the IFN-α levels in the lung were higher in the cranial lung with LPAI infection, although IFN-β levels were higher with HPAI in the caudal lung.[195] This reasons for this difference were not elucidated by the authors. Yet another study showed that HPAI but not LPAI induced increased cytokine expression in chicken dendritic cells.[196]

The effect of P and V gene transfection on cytokine expression did not show a significant difference between the Meredith/02 and Herts 33/56 viruses, apart from when Mx was examined. As expected, the V protein of both viruses reduced the expression of IFN-α and IFN-β when compared with the P protein, although these differences were not statistically significant. The only statistically significant result was seen with Mx after Herts 33/56 V gene transfection, whereby Mx mRNA expression was greatly increased. This result is difficult to interpret, however it is unlikely that the Herts 33/56 V gene itself has

directly influenced the increased expression, rather that transcription of the Mx gene is so sensitive to interferon induction that it has 'escaped' suppression and a meaningful result has not been obtained.

There are a number of limitations to this study that include the fact that the work was carried out *in vitro* and that only a limited range of cytokines were examined. This work was conducted in cell culture using DF-1 cells. DF-1 cells are an immortalized chicken embryo fibroblast cell line and were chosen because they facilitate NDV replication and are relatively easy to propagate. However, fibroblasts are not a preferential cell type for NDV replication *in vivo*. As seen in Chapter 4, the Meredith/02 virus appears to replicate initially in the epithelial cells associated with sites of inoculation, along with mononuclear cells (lymphocytes and macrophages). Herts 33/56 also has a strong tropism for lymphoid tissue. Therefore, whilst these viruses are able to replicate in fibroblasts, they may not exhibit the same cytokine response as would be found in the live bird. In addition, whilst GFP was used as an indicator of protein expression in the DF-1 cells, individual Western blots were not performed for each of the proteins to definitively prove that the proteins had been expressed.
It would also be interesting to examine other cytokines that play a key role in antiviral immunity such as IFN-γ. IFN-γ is a class II interferon which is predominantly expressed in lymphocytes and has been found to decrease the pathogenicity of NDV in vivo.[197] It was not included in this study as it was unlikely to be expressed highly in DF-1 cells.

The aim of this work was to investigate whether the P and/or V genes of the Meredith/02 and Herts 33/56 viruses may account for their difference in pathogenicity. The study was conducted as an *in vitro* pilot trial to investigate whether further *in vivo* work with a greater range of cytokines was warranted. Our transfection studies did not suggest that the proteins significantly differ in their effect on innate immunity. However, the increased induction of Mx mRNA expression by the Meredith/02 virus is an interesting result that requires further investigation.

6.5 Conclusions

This study has shown that the two NDVs Herts 33/56 and Meredith/02 are able to induce the production of type I interferons (IFN-α and IFN-β) and the Mx protein in DF-1 cell culture. There was no significant difference in the level of interferon induction between the virus isolates, however the induced expression of the Mx protein did vary. More Mx protein was expressed when cells were infected with the Meredith/02 virus compared with the Herts 33/56 virus. This increased expression of Mx may lead to a greater inhibition of replication for this virus, thereby decreasing its pathogenicity in infected birds as seen experimentally and in the field.

The ability of the V genes of each of the viruses to antagonize interferon was confirmed but again, there was no significant difference in the degree to which the Herts 33/56 and Meredith/02 viruses could achieve this.

CHAPTER 7
A VIRULENT AUSTRALIAN NEWCASTLE DISEASE VIRUS WITH AN ATTENUATED PHENOTYPE HAS A STEEPENED TRANSCRIPTION GRADIENT

7.1 Introduction

Previous chapters have examined an Australian Newcastle disease virus (NDV) isolate, Meredith/02, which displays minimal pathogenicity in chickens, despite containing a virulent fusion protein cleavage site according to the OIE definition.[6] It is therefore assumed that there may be other molecular determinants that influence the virulence of this virus, other than the cleavage site.

As seen in Chapter 5, there are numerous amino acid variations throughout the genomes of the Herts 33/56 and Meredith/02 viruses, of which none are in regions of known importance to pathogenicity. This makes it difficult to identify any particular genes that may have a significant role in pathogenicity. The previous work in Chapter 6 focused on the V protein's effect on innate immunity but did not show any clearly pertinent differences between the Herts 33/56 and Meredith/02 viruses that may influence pathogenicity. Therefore, an analysis of the transcription gradient of both viruses may provide a broader insight into which genes could be targeted for further investigation into their role in pathogenicity.

NDV, like all paramyxoviruses replicates after transcription of viral mRNA from genomic RNA. Transcription of the negative sense RNA strand occurs within the cytoplasm and primarily involves the nucleocapsid protein (N), phosphoprotein (P) and large polymerase protein (L). Each nucleocapsid protein binds to six nucleotides of the viral RNA, following the rule of six. The subsequent attachment of the P and L proteins forms the ribonucleoprotein (RNP) complex.[174] The viral RNA-dependent RNA polymerase (RNAP) then transcribes the genome in the direction from the 3' leader (promoter) sequence to the 5' end, using a start-stop mechanism which terminates at the end of each gene and begins again at the start of the next gene.[75] However, this process is not entirely efficient and occasionally the RNAP does not bind to subsequent genes. As a result, the downstream

mRNAs are not transcribed and so the mRNAs are not produced in equimolar amounts. This leads to the formation of a gradient of transcribed mRNAs such that more nucleoprotein mRNAs are produced as compared with large polymerase mRNAs, as seen in Figure 7.1. After transcription, the viral mRNAs are translated into the individual viral proteins.

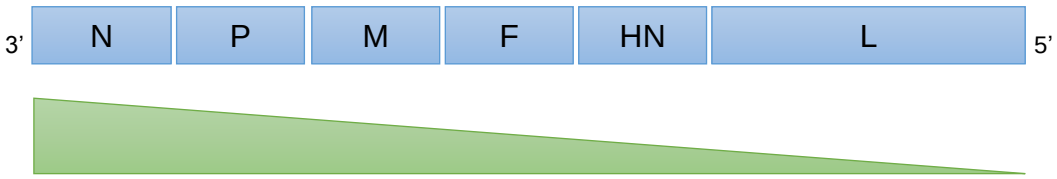


Figure 7.1 Gradient of transcription. Transcription of the NDV RNA genome occurs in a 3' to 5' direction with more mRNA transcripts produced from genes closer to the 3' end.

It is possible to quantify the mRNAs produced by each gene of a virus by quantitative reverse transcriptase PCR (qRT-PCR) or agarose gel electrophoresis to create a transcription gradient for the virus isolate. The profile of the gradient may then be compared between different isolates of NDV to investigate whether there is any correlation between the production of mRNAs and the virus phenotype. Previously, quantification of the viral mRNAs of NDV has been studied using gel electrophoresis via Northern blotting.[198] However, electrophoresis is a relatively less sensitive method of mRNA quantification and so this study will investigate the transcription gradient of NDV using qRT-PCR.

The transcription gradients of a number of other paramxyoviruses, including measles, Sendai virus and Hendra virus have previously been described.[199-201] In brains infected with measles virus, the transcription gradient of the viral mRNAs varied with the type of infection, with persistent infections showing a more shallow transcription gradient compared with the steeper curve of active infections.[199]

The transcription gradient has also been found to vary between different strains of the same virus in the case of vesicular stomatitis virus, another negative-sense RNA virus.[202] In this case, a small plaque phenotype, attenuated strain was found to have a steepened transcription gradient and less viral mRNA transcription overall, when compared with the wild type virus.

Therefore it is hypothesized that there may be differences in the transcription gradients of the Meredith/02 and Herts 33/56 viruses which could help to explain the difference between these viruses in their pathogenicity for poultry.

7.2 Materials and Methods

7.2.1. Infection of DF-1 cells

DF-1 cells were grown to confluency as described in Chapter 3.2.1. 24-well plates were seeded with 5 x 10^4 cells per well in 1 ml growth media. The cells were grown to approximately 95% confluency over 48 hours and then infected with either Herts 33/56 or Meredith/02 virus at an MOI of 0.1. Cells were infected in triplicate or left uninfected as controls. The cells were harvested at 0, 6, 12 and 24 hours by removal of media and the addition of 140 µl of MagMAX lysis buffer. Lysed cells were then stored frozen at -80^{o}C immediately. A 24 hour infection period was chosen as it has been shown with measles virus that RNA and mRNA accumulates rapidly over the first 24 hours of replication and is relatively stable thereafter.[203] Therefore any differences in transcription between virus isolates should be detectable within this timeframe.

7.2.2 RNA isolation

RNA was isolated as per Chapter 3.8 using the MagMAX express viral RNA isolation protocol (Ambion) on the Kingfisher Flex. RNA was eluted in 60 µl of elution buffer and stored frozen at -80^{o}C.

7.2.3 cDNA Synthesis

Two-step RT-PCR was used to quantify each of the mRNA viral gene transcripts. cDNA was synthesized using the SuperScript® III First-Strand Synthesis SuperMix. Oligo(dT)$_{20}$ primers were used to amplify the mRNA transcripts. Oligo(dT)$_{20}$ primers were chosen as they bind to the poly(A) 3' tail of mRNA and will not transcribe the genomic RNA. Reactions were carried out in 24 µl volumes in 0.2 mL thin-walled PCR tubes using oligo(dT)$_{20}$ primer (1 µl, 50 µM), annealing buffer (1 µl), nuclease-free water (8 µl) and template RNA (2 µl). Tubes were heated to 65^{o}C for 5 minutes using a GeneAmp PCR System 2400 (Perkin Elmer), then placed on ice for at least 1 minute. Next, the 2X First-Stand Reaction Mix (10 µl) was added to each tube, along with the SuperScript III®/RNaseOUT™ Enzyme Mix (2 µl). The tubes were then heated to 50^{o}C for 50 minutes

and reactions terminated at 85ºC for 5 minutes. Finally, samples were held on ice before use or stored at -20ºC. cDNA was diluted in RNAase free water using a 1 in 10 ratio before use in the PCR.

7.2.4 mRNA gene transcript primer design

Primers were designed for each gene (N, P, M, F, HN and L) using Geneious software as per Chapter 3.15 and a list of the primers used can be found in Appendix 1, Table A5. The primers were aligned to both the Herts 33/56 and Meredith/02 viruses to confirm that there were no mismatches with either virus. The primers were located within 1,200 bp of the 5' end of each gene, to ensure that any mRNA that was not fully transcribed during the reverse transcription step was still detected by the primers. β-actin was used as an endogenous control rather than the previously used ribosomal RNA because the oligo(dT) primer requires a polyadenylated sequence to bind which is not present on 18S rRNA or 28S rRNA. β-actin has also been shown to be one of the most stable genes for use as an endogenous control.[204, 205] Previously published β-actin primers were used.[185]

The location of the primers in relation to the NDV genome can be seen in Figure 7.2.

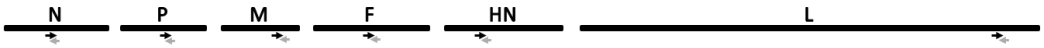


Figure 7.2 Primer pair locations for NDV mRNA amplification. Forward primers are represented by black arrows and reverse primers by grey arrows.

7.2.5 SYBR green PCR

Quantitative PCR was performed using the SYBR® Green PCR Master Mix (Applied Biosystems). Reactions were undertaken in 10 µl, using 2X SYBR Green PCR Master Mix (5 µl), forward primer (0.2 µl, 200 µM), reverse primer (0.2 µl, 200 µM), nuclease-free water (3.6 µl) and template cDNA (1 µl).

Cycling conditions were as follows:
95ºC for 10 min
45 cycles of 95ºC for 15 sec and 60ºC for 1 min
Melt curve: 95ºC for 15 sec, 60ºC for 15 sec, 95ºC for 15 sec
Melting curves were analysed to assess for non-specific amplification.

7.2.6 Standard curve generation

Standard curves for each primer pair were generated by creating six, 10-fold dilutions of cDNA and PCR reactions performed as above in Chapter 7.2.5. The efficiencies of the reactions were calculated by plotting the log of each template dilution series on the x-axis and the C_T value on the y-axis. A line of best was then used to determine the slope. The efficiency (E) of the reaction was then obtained using the equation:
E = 10^(-1/slope).

7.2.7 Data analysis

The comparative C_T method was used to express fold changes of each gene relative to the N gene as per Chapter 3.9.2 with all samples normalised to the endogenous β-actin control.

Any outliers were identified from each of the triplicate technical replicates and removed from the analysis. The average of each group of technical replicates was determined and used to find the mean and standard deviation of the three biological replicates for each target sample. The comparative C_T and subsequent fold change was then determined according to the Applied Biosystems calculations.[206]

7.3 Results

7.3.1 Optimisation and specificity of the polymerase chain reaction

After infection with Herts 33/56 and Meredith/02 and harvesting of the DF-1 cells at 6, 12 and 24 hours, the RNA was extracted and reverse transcribed into cDNA. Standard curves were generated by SYBR green PCR for each of the primer pairs using ten-fold dilutions of the cDNA from a 12 hour Herts 33/56 sample. The efficiencies of each reaction were then calculated and can be seen in Table 7.1.

Gene target	Efficiency
N	2.0
P	1.9
M	1.9
F	1.9
HN	2.0
L	2.1
β-actin	1.8

Table 7.1 Efficiency of primer pairs for each gene target.

The efficiencies were all within the range 1.8x to 2.2x, allowing the comparative C_T method to be used for comparison.[207]

Melting curves were analysed for the specificity of the PCR reactions and the associated plots can be seen in Figure 7.3. All melting curves displayed single peaks indicating that only the target sequence had been amplified.

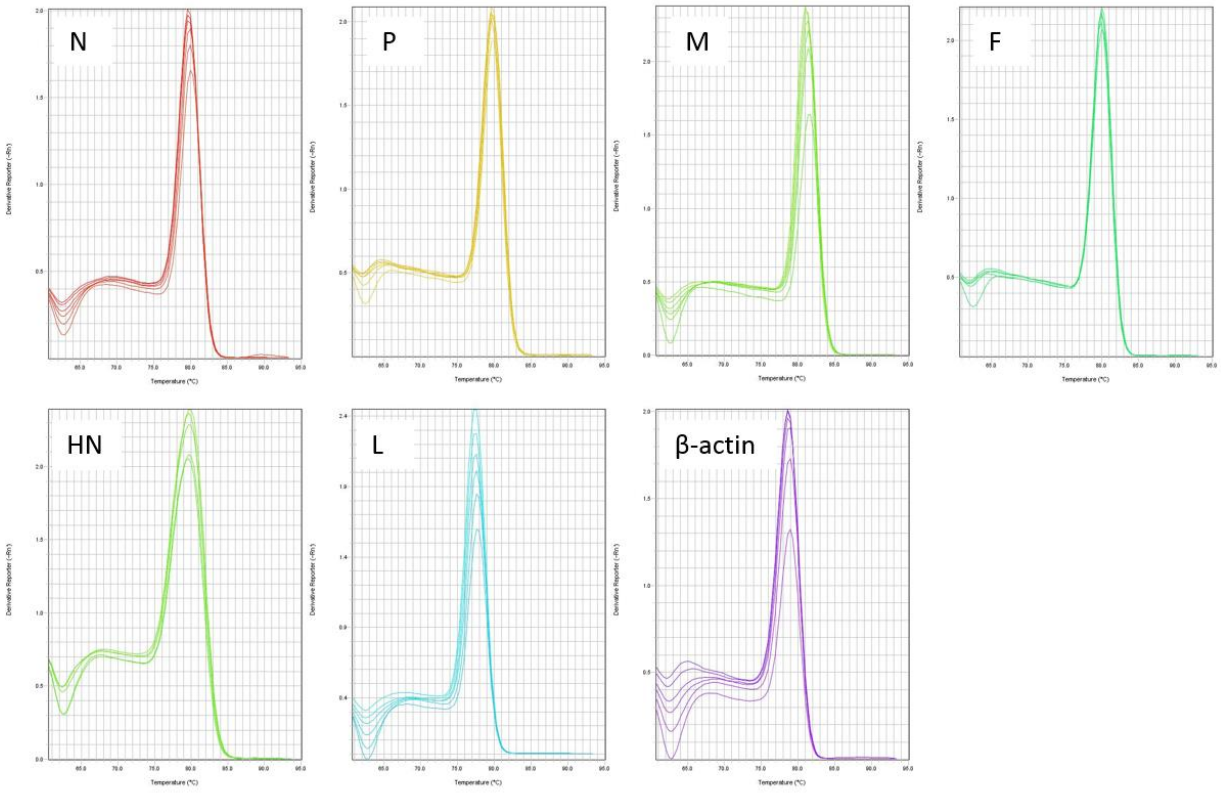


Figure 7.3 Melting curve analysis for each of the 7 SYBR green PCR reactions

Any wells showing abnormal amplification curves or unusual melt curves were omitted from the analysis. In all cases where samples were omitted, only one of the three technical replicates was affected. For all PCR plates, there was no amplification detected in the negative control wells.

7.3.2 Transcription gradients

The fold changes for the individual genes of the viruses were then calculated and the transcription gradients were plotted as seen in Figure 7.4. In addition, the ratio of mRNAs for each virus can be seen in Table 7.3. The curves show a progressive decrease in the detection of the mRNA transcripts of each gene moving from the 3' end of the genome to the 5' end. The cell cultures infected with either virus contained a much lower level of large polymerase gene transcription compared with the nucleocapsid gene.

At 6 hours, the transcription gradients were relatively similar between the two viruses, however the gradient between the N and P transcripts was steeper for the Meredith virus, indicating that transcription of the Meredith/02 P gene is relatively less efficient. At 6 hours, the fold change for the Herts 33/56 P gene mRNA was 1.8 times that of the Meredith/02 mRNA. At 12 hours a similar pattern is present, although the difference between the two gradients at the point of P gene transcription was even greater with the Herts 33/56 P gene transcript 3.2 times that of the Meredith P gene. Once again, at 24 hours the Meredith virus showed a steeper gradient, particularly between the N and P gene mRNA transcripts, with the Herts 33/56 P gene mRNA 14 times greater than the Meredith P gene mRNA. In all cases, at the point of the M gene transcript, the gradients of the curves flattened significantly and there was little difference between the fold change of the M mRNA and L mRNA (relative to the N gene mRNA). As seen in table 7.2, at 24 hours, the proportions of transcribed mRNAs downstream from the nucleocapsid gene were minimal for the Meredith/02 virus with a ratio of mRNA of 100:3:2:1:3:1 (N:P:M:F:HN:L). In contrast, the equivalent ration for Herts 33/56 was 100:42:18:14:14:8.

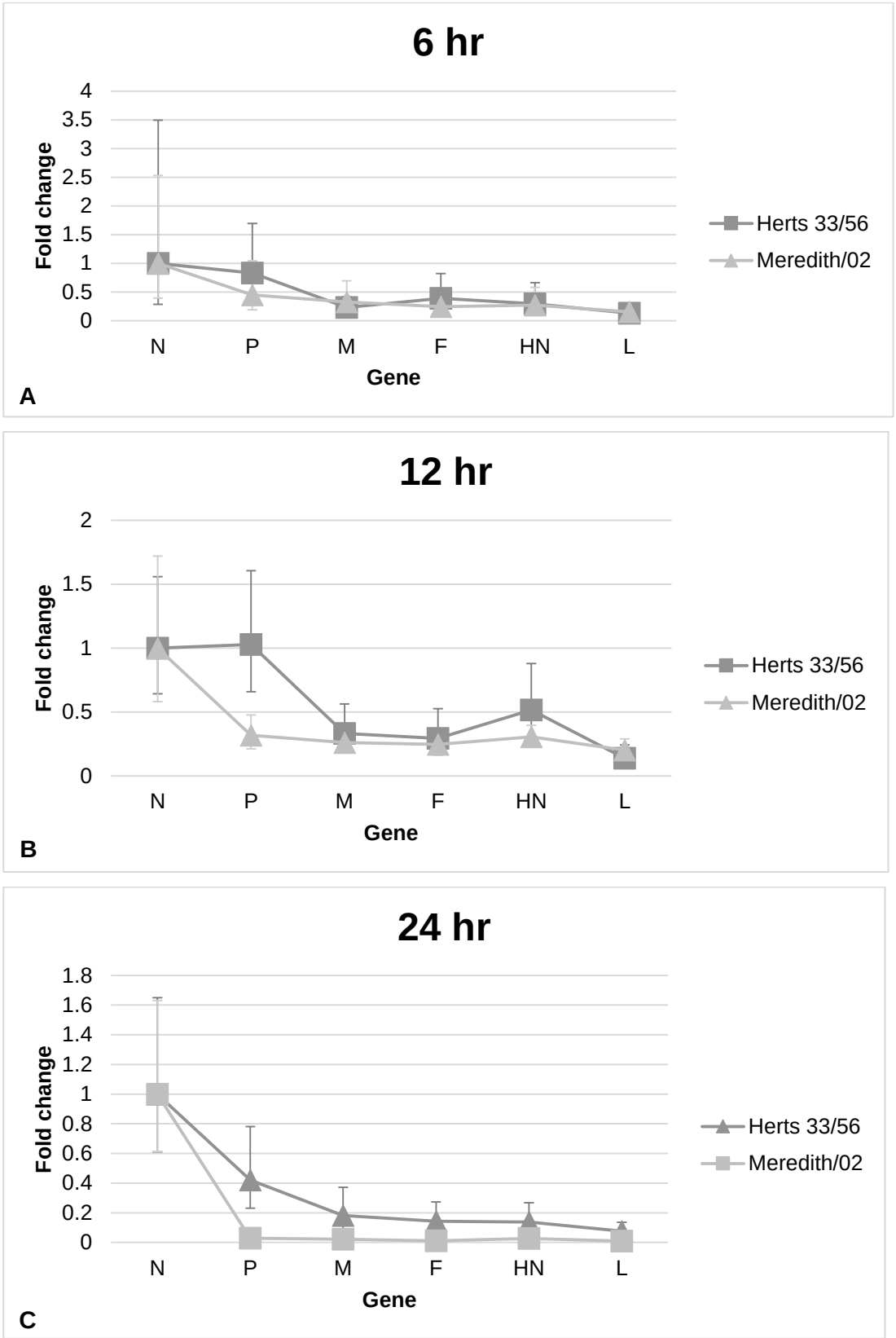


Figure 7.4 Relative quantification of mRNA transcripts of NDV genes after infection of DF-1 cells with Herts 33/56 or Meredith/02 viruses at time points 6 hours (A), 12 hours (B) and 24 hours (C). Each gene mRNA is expressed as the fold change relative to the N gene transcript. Data were normalised to β-actin mRNA. Error bars represent the standard deviation of the mean of 3 replicates.

Time (hrs)	mRNA ratio (N:P:M:F:HN:L)	
	Herts 33/56	Meredith/02
6	100:83:23:39:29:13	100:45:33:24:27:15
12	100:103:33:29:41:14	100:32:26:25:30:20
24	100:42:18:14:14:8	100:3:2:1:3:1

Table 7.2 Ratio of transcribed mRNAs for each gene, expressed as a percentage relative to the N gene transcript.

7.4 Discussion

This study investigated the transcription gradients of two NDVs which share a common, virulent fusion protein cleavage site but which vary in pathogenicity. The Herts 33/56 virus is a highly pathogenic viscerotropic, velogenic virus, whereas the Meredith/02 virus only produces mild clinical signs in infected chickens. It has therefore been hypothesized that areas of the genome other than the fusion protein cleavage site must be responsible for the reduced pathogenicity of the Meredith/02 virus. It was thought that significant differences in the transcription of the viral genes may help to explain the pathogenicity of the viruses. Therefore, the transcription gradients of both viruses were constructed via qRT-PCR and compared.

The overall slope of the transcription gradients presented in Figure 7.4 are consistent with what is known about the rate at which the genomes of RNA viruses are transcribed. It is well established that transcription is progressively attenuated as the polymerase moves from the 3' to 5' end of the viral RNA genome and this was confirmed for both the Herts 33/56 and Meredith/02 viruses in this study. Previous work by Collins, et al. using the virulent NDV-AV strain also showed that transcription of NDV genes occurs in non-equimolar amounts with molar ratios of 100:65:41:33:3 corresponding to N:F:M:HN:L.[198] These ratios are relatively similar to those found in this study, apart from with Meredith/02 at 24 hours. At 24 hours, the Meredith/02 virus had a ratio of mRNA transcription of 100:3:2:1:3:1. This means that after transcription of the N gene had occurred, only minimal amounts of the remaining genome were transcribed.

The substantially increased steepness in the transcription gradient between N and P for Meredith/02 at 24 hours is interesting. An increased gradient can also be seen for Herts

33/56 at 24 hours, compared with the earlier time points, albeit not to the same extent as with Meredith/02.

Presumably by 24 hours, the rate of viral replication and transcription have started to decrease due to availability of cellular components in the cell culture system. However, this does not explain the difference between the proportion of P gene transcription by Herts/33 and Meredith/02. Perhaps the RNAP of the Meredith/02 virus is more easily inhibited at an earlier time point than the RNAP of the Herts 33/56 virus. Another paramyxovirus, Hendra virus has been shown to have a steepened transcription gradient at 24 hours at the matrix-fusion protein junction as opposed to the nucleocapsid-phosphoprotein junction as in this case.[208]

It is likely that the N, P and L proteins which comprise the viral replication complex have influenced the rate of transcription of the Meredith/02 virus. The ratio of proteins within the viral replication complex is an important factor in the efficient replication for many viruses.[209] The ratio of N, P and L mRNAs at 6 hrs is 100:83:13 for Herts 33/56 compared with 100:45:15 for Meredith/02. There is therefore, a 2 fold difference in the proportion of P mRNA transcription between the viruses, perhaps limiting the efficiency of transcription of the Meredith/02 phosphoprotein and hence impairing replication complex formation.

At the molecular level, there may be many reasons for these differences in transcription ratios. The gene-start, gene-end and intergenic sequences between the individual genes help to modulate transcription but are not functional during virus replication.[74, 210] However, it was shown by Yan et al. that altering the length of the intergenic sequence of NDV can attenuate downstream transcription.[153]

There are very few studies which examine the transcription gradients of virus strains which vary in pathogenicity. However, the work investigating the steepened transcription gradient of an attenuated vesicular stomatitis virus (VSV) is consistent with our findings, in that the steepened gradient of Meredith/02 transcription is also associated with reduced pathogenicity.[202] As hypothesized in the VSV study, it is also possible that there is a mutation in the Meredith/02 polymerase complex that prevents it from efficiently reinitiating transcription after the initial nucleocapsid protein mRNA has been transcribed.

When the sequences of the gene boundaries of the nucleocapsid and phosphoprotein were compared in Chapter 5.3.3.8, there were no differences between the Meredith/02 and Herts 33/56 viruses. Thus, it may be more likely that the differences in transcription reinitiation are due to variations in the large polymerase protein itself.

It would also be interesting to extend this study to include additional ND viruses with varying pathogenicity for chickens, to investigate whether all less pathogenic viruses have an increased transcription gradient.

These findings provide further evidence that regions of the NDV genome other than the fusion protein cleavage site may influence pathogenicity, particularly in the context of Australian ND viruses.

7.5 Conclusions

This study has shown that the mesogenic Australian Meredith/02 NDV has a steepened transcription gradient when compared with the virulent Herts 33/56 virus. This steepened transcription gradient may indicate altered transcription of the Meredith/02 virus which could be related to components of the viral replication complex, in particular, the large polymerase protein. However, further investigation is required to determine whether this is a consistent finding associated with the pathogenicity of other NDVs.

CHAPTER 8
GENERAL DISCUSSION

8.1 Introduction

The overall aim of this thesis has been to investigate the molecular basis of pathogenicity of Newcastle disease virus (NDV) in chickens. Newcastle disease (ND) can have significant impacts on poultry production, particularly in areas in which it is endemic, such as throughout Asia, Africa, the Middle East and parts of Central and Southern America. However viruses such as NDV can be difficult to control because of their wide variability in pathogenicity and broad host range. This variability in pathogenicity has led to certain criteria being developed to assess whether a virus is likely to cause significant disease. These criteria are primarily based on the molecular sequence at the fusion protein cleavage site, with a multiple basic amino acid motif indicating a virulent (velogenic or mesogenic), OIE notifiable virus.[6]

This work focused on an Australian NDV which caused an outbreak of ND in Meredith, Victoria in 2002. In this case and in other Australian outbreaks from 1998-2002, some of the veterinarians and poultry experts involved with investigating and controlling the disease at the time, found it difficult to believe that the clinical disease seen in the field was associated with a virulent NDV.[9, 56] These Australian NDVs, whilst fitting the OIE definition of ND, did not appear as pathogenic in the field as expected. However, as per Australian guidelines for the control of ND, the flocks were culled in an attempt to eradicate the virus.

This work has attempted to further characterize the Meredith/02 NDV and to identify whether there are areas of the viral genome, other than the fusion protein cleavage site that may contribute to the decreased level of pathogenicity. It was thought that if there were other markers of pathogenicity, in conjunction with the fusion protein cleavage site, they could be used to predict whether a virus may be somewhat less pathogenic, which may then lead to the use of alternative control methods, as opposed to culling.

This work comprises four main components to investigate the pathogenicity of the Meredith/02 virus; comparative pathogenicity in an experimental setting, virological characterization, the ability of the Meredith/02 virus to both induce expression of and

antagonize components of the innate immune system and comparative transcription gradients.

The key findings from each of these pieces of research will be discussed, along with overall conclusions and suggestions for future directions that research in this area could take.

8.2 Comparative pathogenicity

The purpose of the research conducted in Chapter 4 was to compare the pathogenicity of four NDVs for poultry in an experimental setting, using two virulent viruses that are exotic to Australia and two Australian viruses, including the Meredith/02 virus. It was hypothesized that the Meredith/02 virus would not induce severe clinical signs in the challenged birds despite being classified as a virulent virus. It was important to be able to observe the effect of the virus in a laboratory setting, using SPF birds that had not been exposed to other pathogens that could alter the expression of the disease. The aim therefore, was to replicate what was seen in the field situation, however with controls and standard inocula to enable comparisons of pathogenicity and pathogenesis, including tissue tropism and degree of virus replication.

The Peats Ridge/98 virus was included in the work because it was an avirulent virus that was determined to be the precursor to the Australian virulent viruses with only two nucleotides difference at the fusion protein cleavage site.[71] The Herts 33/56 virus and Texas GB viruses were included because these viruses were known to represent the two velogenic pathotypes; viscerotropic and neurotropic respectively.

It was found that the Meredith/02 virus had exactly the same fusion protein cleavage site as the velogenic viscerotropic Herts 33/56 virus. However, as per the field situation, it displayed minimal clinical signs in chickens. Only 2 out of 6 birds showed mild depression and increased respiratory effort on day 3 post inoculation and these signs resolved within hours. This is unusual although not without precedent, as pigeon paramyxoviruses with virulent cleavage sites have also showed similarly minimal clinical signs in chickens as described in a review by Dortmans et al.[3] In addition, other mesogenic viruses, can also be less pathogenic in an experimental setting than their fusion protein cleavage site motif would suggest. In a similar experiment using the mesogenic Roakin and Anhinga viruses,

there were no clinical signs in the infected birds, although splenic pathology was noted grossly and histologically.[98] Those two mesogenic viruses also had multibasic cleavage sites of [112]RRQKRF[117], as per the Texas GB virus, with the Roakin virus able to induce moderate mortality in poultry.[211] The Anhinga virus was originally isolated from a dead Anhinga (darter) from a Florida zoo, however whether the mortality event was attributable to infection with NDV is unclear.[212]

Given the importance of the presence of a polybasic fusion protein cleavage site to the definition of ND, the ability to correlate the sequence motif with clinical signs, particularly in the field situation is significant. Whilst the laboratory setting is useful in providing baseline data for comparison, it does not entirely replicate a commercial poultry enterprise with the additional stressors of increased housing density and bacterial, viral and parasitic pathogens, amongst others. Therefore, it should not be assumed that because a virus was virtually apathogenic experimentally, that it will behave similarly in a poultry flock.

Whilst the key focus of this piece of research was to evaluate pathogenicity, pathogenesis of these four viruses was also investigated. Explaining where and how the virus replicates within the host, can help to understand why one virus is more pathogenic than another and may provide future direction for molecular based pathotyping. In this study, two birds were euthanased on both days 2 and 4 post inoculation to assess any early viral replication. The Peats Ridge/02 virus was restricted to replicating on epithelial surfaces at sites of inoculation, limiting the clinical signs seen. However, whilst the Meredith/02 virus was able to spread systemically to lymphoid tissues such as the spleen early in the infectious process, the amount of virus present in these tissue was significantly less than with standard velogenic viruses. This capacity to infect lymphoid tissue indicates that the fusion protein of the Meredith/02 virus is able to be cleaved by furin-like proteases as the cleavage site suggests. This replication in lymphoid tissue predominantly associated with macrophages, which is well recognized for virulent NDV.[98] The Meredith/02 virus however, did not replicate within neural tissue experimentally, thereby limiting its pathogenicity. By comparison, the Herts 33/56 and Texas GB viruses did show antigen staining in the CNS. At the time of its isolation, a small number of birds infected with the Meredith/02 virus were observed with mild nervous signs, so presumably, the virus is capable of replicating in nervous tissues given the appropriate circumstances.

Overall, the Meredith/02 virus was not able to replicate to the same degree as the Herts 33/56 and Texas GB viruses and this was associated with a lesser degree of both quantity and distribution of antigen staining by immunohistochemistry. These observations could be explained by host factors, such as the immune response to the Meredith/02 virus suppressing replication, or could be an intrinsic element of the virus itself, limiting key elements of virus entry, replication and budding.

It would have been useful to be able to examine tissues from birds from the original outbreak, to compare lesion and antigenic distribution with the experimental birds. However, only a minimal number of poorly preserved specimens were available and so were not included in the analysis.

Future work in this area would be useful to expand the range of Australian viruses used to investigate pathogenicity, particularly with additional viruses from the Mangrove Mountain outbreaks in 1998-1999. During these outbreaks a range of clinical signs were noted, however the affected birds were also concurrently infected with agents such as *Pasteurella multocida* and Mareks disease virus.[56] Therefore, assessing the pathogenicity of these viruses in SPF birds may lead to a clearer picture of how these viruses compare with each other. Subsequent to this, whole genome sequence examination could be undertaken to identify sequence changes associated with the pathogenicity. Similar work examining sequence variations in the NDVs from the 1998-2002 outbreaks has already been undertaken, however much of the work was purely based on molecular data without the incorporation of clinical signs and pathological lesions.[53, 71]

8.3 Virus characterization

Following on from the pathogenicity research in Chapter 4, it was necessary to characterize the Meredith/02 in more detail using standard virological techniques. In this way the growth characteristics of the virus could be evaluated and used as baseline measures for further experimental work. Again, the same four viruses were used to examine virus growth in cells and eggs, to calculate the mean death time in eggs, to sequence the whole genome of each of the viruses and to investigate virus replication within the chicken embryo. After completing the comparative pathogenicity work, it was thought that the Meredith/02 virus could be classified as either a mesogenic or lentogenic pathotype. Further clarification of the pathotype would then allow for a better comparison with other known viruses with similar characteristics.

The mean death time in eggs is one of the most well recognized methods for determining the pathotype of ND viruses. The MDT for the Meredith/02 virus was calculated at 68 hours which classifies it as a mesogen. The Herts 33/56 and Texas GB viruses were both velogenic with MDTs of 44 and 60 hrs respectively and the Peats Ridge/98 virus was found to be lentogenic with an MDT of 116 hrs. The MDT can also be compared with the ICPI, another measure of pathogenicity. However, given that the ICPI represents an unnatural route of infection and that strong justifications are required for its use from an ethical perspective, it was not undertaken in this work. Whilst the ICPI often correlates with the fusion protein cleavage site and the pathogenicity of a virus, it does not predict pathogenicity in cases. Previous work completed at AAHL, showed that the ICPI of the Meredith/02 virus was 1.61, indicative of virulence. Mesogenic viruses such as Roakin also have similar ICPIs of >0.7.[213] Therefore whilst pathotypes do not have strict boundaries and there is often some overlap in characteristics of various ND viruses, it appears that the Meredith/02 virus should be considered a mesogen. Given that other viruses from the 1998-2002 outbreaks have previously been reported as velogenic it would be useful to clarify the pathotype of these viruses as well, using the MDT along with experimental infection in poultry.[104] It is interesting to note that in some countries where ND is endemic, mesogenic viruses are used as vaccines (R2B, Roakin, Mukteswar and Komarov viruses), however they are not acceptable in the Australian situation.[6, 83, 214]

The whole genome sequences of all four viruses were constructed and analysed with reference to previous sequencing work. Important structural features of the viruses were identified and compared when aligned. Whilst there were no obvious sequence differences detected to account for the variations in pathogenicity between the viruses (apart from the F cleavage site of Peats Ridge/98), knowledge of the whole genome sequences could be used to inform further work, such as cloning of individual genes and construction of reverse genetics systems. The P gene was shown to be the most variable across all four viruses at both the nt and aa level and as such was identified as a potential gene for further analysis. The HN gene was the second most variable gene and the unique 9 aa extension of the Australian viruses could be a target for future investigations, although previous studies have given conflicting results on the importance of the HN gene in pathogenicity (as reviewed Chapter 2.8.3).

During the initial outbreaks of ND in Australia in 1998, egg inoculation was used to assess the virulence of the virus (Deborah Middleton, pers. comm.). Embryos at 9-11 days old

were inoculated with virus via the allantoic route and the chorioallantoic membrane and embryos examined by immunostaining for virus antigen. Virulent viruses were able to replicate on both sides of the chorioallantoic membrane and throughout the developing embryo itself. Avirulent or lentogenic viruses were restricted to replicating on the allantoic surface of the CAM. This procedure was repeated with our four viruses and it was shown that the Meredith/02 virus was able to replicate throughout all surfaces of the CAM and that antigen was widely distributed throughout the internal organs of the developing chicken embryo. This indicates that furin-like proteases are able to cleave the fusion protein at the multibasic amino acid cleavage site as per other virulent NDV strains. Therefore the cleavability of the fusion protein of the Meredith/02 virus does not appear to be the limiting factor for pathogenicity. This finding is supported by the previous pathogenicity work in Chapter 4 that showed virus replication in the spleens and caecal tonsils of birds infected with Meredith/02 virus.

Whilst this section of work was not able to identify any particular genes or molecular sequences that could explain the pathogenicity characteristics of the Meredith/02 virus, it provided a useful dataset for further molecular analysis. It has also classified the Meredith/02 virus as a mesogen which allows comparisons to be drawn with other mesogenic ND viruses.

8.4 Innate immune system

Following on from the virus characterization work, it was important to narrow the focus of study to particular genes which may influence pathogenicity. The V protein was chosen to investigate because of its role in antagonism of components of the innate immune system during NDV infection.[11] The P gene encodes both the P protein and V protein as a result of RNA editing, therefore both proteins were included in the study. It was hypothesized that the Meredith/02 virus may not be able to antagonize the innate immune system of the host effectively, leading to a decreased ability to replicate within avian cells.

This study comprised two parts; initially the mRNA expression of interferon-α, interferon-β and the Mx protein was assessed by qRT-PCR after infection of DF-1 cells with either the Meredith/02 or Herts 33/56 virus. Secondly, P and V proteins from each virus were expressed in DF-1 cells and their ability to antagonize interferon and Mx mRNA expression was evaluated. Both of these viruses could induce transcription of IFN-α, IFN-β and the

Mx genes, indicating that the DF-1 cell line could be used effectively to assess the antagonistic effects of the P and V proteins. The levels of interferon gene expression were similar between both viruses, however more Mx mRNA was expressed with Meredith/02 infection compared with infection with Herts 33/56.

The results from the P and V protein antagonism experiment didn't appear to show any significant differences between the two viruses in the degree to which interferon could be antagonized. However, the V proteins of both viruses were able to antagonize interferon to a greater level than the respective P proteins, which helped to validate the experimental methodology. There was one anomaly in this work however, in that the Mx mRNA was seen to be significantly more expressed when the Herts 33/56 V protein was transfected into the cells compared with transfection with the Herts 33/56 P protein. This was unable to be explained from a virological perspective. However, it appears that the Mx protein is particularly quick to respond to stimulation and can be transcribed at very high levels (Daniel Layton, pers. comm.). Therefore, it is possible that in this experimental work, including the initial expression work with the Meredith/02 virus, that Mx mRNA expression was too responsive to stimulation to meaningfully evaluate.

Overall, it was not possible to show that the V protein of the Meredith/02 virus was able to antagonize the innate immune system to a greater degree than the Herts 33/56 virus using the cytokines that were included in this work. However, it would be beneficial to evaluate a broader set of innate immune system cytokines to fully validate this conclusion. Given that that the innate immune system involves a complex network of interacting cytokines, it would be particularly useful to use techniques such as RNA-Seq to profile cytokine gene expression on a larger scale. However, the cost of such analysis was prohibitive for this project.

8.5 Transcription gradient

Given that previous work had not readily identified a specific protein or molecular sequence of interest to evaluate for its effects on the pathogenicity of the Meredith/02 virus, a broad approach to investigating the individual viral genes was taken. This involved analyzing the transcriptional gradient of two of the viruses in this study; Herts 33/56 and Meredith/02. It was hypothesized that significant differences in the transcription gradients of these two viruses could be associated with differences in the ability of the viruses to

transcribe their genomes and thereby replicate. A steeper gradient would imply less efficient transcription.

After construction of the transcription curves, it was shown that the Meredith/02 virus did have a steeper gradient than the Herts 33/56 virus, particularly between the N and P gene mRNA transcripts. Whilst the V protein was not specifically examined as part of the transcription gradient, it was assumed that transcripts encoding the V protein would be expressed in similar amounts to the P gene mRNA. However, further work would be required to investigate this in more detail.

Other work investigating the transcription gradients of Sendai, Hendra and measles viruses have also shown transcriptional attenuation at certain gene boundaries. The Sendai virus shows attenuation at the M-F and HN-L boundaries, Hendra virus at the M-F and G-L boundaries and measles virus at the N-P and H-L boundaries.[200,199,201] The attenuation seen with the Meredith/02 virus was therefore most similar to the measles virus. It was suggested in the case of measles that the decreased numbers of gene transcripts at the 5' end of the genome in persistently infected brain tissue could be associated with impaired budding of virus from the cell. However, the effect of the decreased number of P transcripts could not be explained. Work undertaken by Hodges, et al. draws parallels with this thesis as it also investigated an attenuated vesicular stomatitis virus strain, which was found to have a steepened transcription gradient. It was hypothesized that mutation in the polymerase complex of the vesiculovirus may have resulted in decreased downstream transcription.[215]

This transcription gradient investigation has been limited by the cell types and number of viruses used. It would be helpful to repeat this work in different cell lines to ensure that the transcriptional profiles that were seen were not associated with the DF-1 cell line. In addition, it would be worthwhile to produce transcription profiles of multiple ND viruses, representing all pathotypes, to determine whether the transcription gradient can always be correlated with pathogenicity. This type of correlation has not been reported in the literature as yet.

The genes that play the greatest role in virus transcription and may therefore have influenced the gradient are the N, P and L genes, otherwise known as the viral replication complex. Given that transcription and translation are governed by similar mechanisms it

was therefore hypothesized that these genes may also have a role in pathogenicity of the Meredith/02 virus. All three of these genes have previously been found to be determinants of pathogenicity of the pigeon paramyxovirus.[12] Given that the gradient between the N and P genes of the Meredith/02 virus was the steepest, it could be hypothesized that either the P or L proteins are implicated in the attenuation of transcription. When the sequences at the P gene boundaries were compared, there was no difference between the Meredith/02 and Herts 33/56 viruses. Therefore, the most likely explanation for the reduced reinitiation of transcription at the P gene would be due to differences in the large polymerase protein.

8.6 Further work

After attempting to narrow the focus of the molecular basis of pathogenicity into particular regions of the NDV genome, it was hoped that by using reverse genetics techniques and interchanging specific genes, it would be possible to pinpoint sequences that explain the mesogenic pathotype of the Australian Meredith/02 virus. However, over the time that this work was undertaken, it became apparent that it would be unlikely that identification of such specific regions would be within the scope of this study. Therefore, a broader investigative approach was undertaken.

In other studies such as that conducted by Dortmans et al. using the pigeon paramyxovirus, it has been found that multiple genes contribute to virulence, in their case, the viral replication complex (N, P and L genes).[12] In the context of a virus that contains only six genes, this is still a significant portion of the genome and so pinpointing specific molecular pathogenicity determinants would involve even greater analysis. That is not to say that it is an impossible task however.

Overall, it seems that other than the fusion protein cleavage, which is the primary determinant of pathogenicity, pathogenicity of NDV is most likely a multigenic trait. This makes determining the combination of molecular sequences that lead to overall pathogenicity very difficult. In terms of diagnostic testing and influencing the guidelines that govern the declaration of an ND outbreak, it is important that pathogenicity determinants can be identified quickly and consistently across viruses. It would be necessary to have particular regions of the genome to target diagnostically by qPCR.

Although, as next generation sequencing becomes more routinely incorporated into diagnostic testing, the need for specific qPCR may be reduced somewhat.

It is possible that into the future, reverse genetics approaches may become more streamlined and less cost-prohibitive and such work may be more readily achievable than at present. Certainly, the use of minigenome assays to investigate replication would be a useful initial step. In addition, more sophisticated bioinformatics approaches may be able to identify pathogenic sequences more easily *in silico*. In this context it would be useful to continue to investigate pathogenicity determinants for the Meredith/02 virus and other viruses, especially mesogens with unexplained phenotypes. In particular, following up on the transcription gradient study and with reference to work by Dortmans et al. and Rout et al., it would be very interesting to further examine the large polymerase protein as a pathogenicity determinant.[12, 13]

Whilst this research can't be directly applied to the current status of ND diagnosis in Australia, there have been a number of questions posed during the course of this work that are worthy of discussion. Firstly, if we were to detect ND viruses similar to the Meredith/02 virus in Australia again, would a ND outbreak be declared? Given that the current OIE definition of an ND outbreak is still based on the sequence motif at fusion protein cleavage site, then such a virus would still be notifiable to the OIE and control and eradication measures would be based on the current AUSVETPLAN guidelines.[83] This would involve stamping out, disinfection, quarantine and movement controls.

Secondly, is the OIE definition of a ND outbreak appropriate for mesogenic viruses such as the Meredith/02 virus? Whilst the Meredith/02 virus did not produce the high mortality and morbidity rates of typical velogenic ND viruses, it still caused an egg drop on the affected property. This would cause significant production losses in the context of a layer farm. The potential impact of the virus on broiler production is however, unknown. In addition, it is unclear whether the virus has the capacity to shed extensively, spread easily and potentially mutate to a more pathogenic form. Therefore, in the absence of this data, it would be prudent to continue to include isolates with mesogenic pathotypes as notifiable ND viruses.

8.7 Conclusions

This thesis has furthered our knowledge surrounding the molecular basis of pathogenicity of NDV in the context of an Australian virus isolated in Meredith, Victoria in 2002. This virus has been classified as mesogenic based on its fusion protein cleavage site, MDT and ICPI and is therefore notifiable to the OIE despite causing minimal clinical signs in infected birds both in the field and experimentally. The virus has a decreased ability to replicate systemically within infected poultry when compared with typical velogenic viruses, although it is able to replicate extensively throughout embryonated chicken eggs. At the molecular level, there are a number of sequence differences between this virus and velogenic viruses, however none of these differences occur in regions with known functional importance. Investigations into the induction of innate immunity and antagonism of interferon via the V protein have showed no meaningful differences between the Meredith/02 virus and the velogenic Herts 33/56 virus. However, the Meredith/02 virus has an increased transcription gradient when compared with the velogenic Herts 33/56 virus, with greatest transcriptional attenuation at the N-P junction, implicating the viral replication complex in pathogenicity.

Therefore, this study has further characterized and provided important baseline data on the Meredith/02 virus, and whilst it has not been possible to determine specific molecular sequences are associated with its attenuated phenotype, it has provided options for further research in this area.

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APPENDICES

Appendix 1 Primer and probe sequences

Primer/Probe	Sequence (5' – 3')	Reference
NDV M forward	AGTGATGTGCTCGGACCTTC	[161]
NDV M reverse	CCTGAGGAGAGGCATTTGCTA	
NDV probe	[FAM] - TTCTCTAGCAGTGGGACAGCCTGC - [BHQ]	
18S forward	CGGCTACCACATCCAAGGAA	[216]
18S reverse	GCTGGAATTACCGCGGCT	
18S probe	[VIC] -TGCTGGCACCAGACTTGCCCTC - [TAMRA]	

Table A 1 Real-time TaqMan NDV PCR primers and probes

Primer	Sequence (5' – 3')	Reference
M13 forward	CAGGAAACAGCTATGAC	Applied Biosystems
M13 reverse	GTAAAACGACGGCCAG	Applied Biosystems
454 cDNA random primer	GTTTCCCAGTAGGTCTCNNNNNNNN	[217]
454 amplification primer	cgccGTTTCCCAGTAGGTCTC	[217]

Table A 2 Cloning and Sequence Primers

Primer	Sequence (5' – 3')	Reference*
P gene F 1	GATGTTCCAGATTATGCTATGGCCACCTTTACAGATGCGG	JB
P gene F 2	gcatgaattcgccaccATGTATCCATATGATGTTCCAGATTATGC	JB
P gene R	gcatCTCGAGTTAGCCATTCAGCGCAAGGCGC	JB
Herts 33/56 V F1	gcatgaattc gccaccATGTATCCATATGATGTTCCAGATTATGC	JB
Herts 33/56 V F2	GTCGTCCAATGCTAAAAAAGGGCCCATGGTCGAGCCCTC	JB
Herts 33/56 V R1	GAGGGCTCGACCATGGGCCCTTTTTTAGCATTGGACGAC	JB
Meredith V F1	gcat gaattcgccaccATGTATCCATATGATGTTCCAGATTATGC	JB
Meredith V F2	GTCATCTAATGCTAAAAAGGGGCCCAGTGTCGAGCCCTC	JB
Meredith V R1	GAGGGCTCGACACTGGGCCCCTTTTTAGCATTAGATGAC	JB
Meredith V R2	gcat CTCGAG TTAGCCATTCAGCGCAAGGCGC	JB
pCAGGS forward	GTGCTGGTTGTTGTGCTGTCTC	JB

*Table A 3 Cloning primers for P and V protein expression (*JB: Jemma Bergfeld unpublished)*

Primer/Probe	Sequence (5' to 3')	Reference
IFN-alpha fwd	GACAGCCAACGCCAAAGC	[188]
IFN-alpha rev	GTCGCTGCTGTCCAAGCATT	[188]
IFN-beta fwd	ACAACTTCCTACAGCACAACAACTA	[189]
IFN-beta rev	GCCTGGAGGCGGACATG	[189]
Mx fwd	GTCCAAGAGGCTGAATAACAGAGAA	[190]
Mx rev	GGTCGGATCTTTCTGTCATATTGGT	[190]
28S fwd	GGCGAAGCCAGAGGAAACT	[188]
28 rev	GACGACCGATTTGCACGTC	[188]

Table A 4 SYBR Green PCR Primers for immune genes

Primer/Probe	Sequence (5' to 3')	Reference*
NDV N forward	TATGCAGGAGCGCAATCCAA	JB
NDV N reverse	TTGCGGCCTCTCTTAAGCTC	JB
NDV P forward	CTCTCCGATCAGAGCAGAGC	JB
NDV P reverse	AGACATCATCGCCTGCACAA	JB
NDV M forward	AGTGATGTGCTCGGACCTTC	[161]
NDV M reverse	CCTGAGGAGAGGCATTTGCTA	[161]
NDV F forward	GTAGTGGCCTGATCACCGG	JB
NDV F reverse	CAGGTAGGTGGCACGCATAT	JB
NDV HN forward	GGTTGCACTCGGATACCCTC	JB
NDV HN reverse	ATGTCCGAAGCACACCAAGT	JB
NDV L forward	TCCAGTCCTTTACCGAGACT	JB
NDV L reverse	AAGCTCTCTGCACAGAACGG	JB
β-actin forward	AGAGGCTCCCCTGAACCCCAAAGC	[185]
β-actin reverse	CTGGATGGCTACATACATGGCTGG	[185]

*Table A 5 Transcription gradient SYBR green primers (*JB: Jemma Bergfeld unpublished)*