PARAMYXOVIRUSES IN AUSTRALIAN SNAKES

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BSc, BVMS

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

2012
I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

.................................................................

Tim Hyndman, 2012
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It is with pleasure that I acknowledge the many people that have helped me with this project over the last six years.

Firstly, to my supervisor Phil Nicholls, thank you for your patience and support during this project. I came to you in 2005 with an idea to look for a virus which many believed to be responsible for several outbreaks of disease in Australian snakes... Or as I put it to you initially, a big problem in a little world. Little did I know that hundreds of samples would need to be screened over two and a half years before a virus was finally isolated, and as if this wasn’t enough, another year would be needed before the identity of this mystery virus would be revealed. So Phil, I sincerely thank you for appreciating what this project meant to me and for providing me with the freedom and assistance to pursue this project despite the many challenges that it presented.

A special thank you also needs to go to the many others that helped me at various stages of this project. To Rachel Marschang, you helped me more than anyone while I was learning the art of classical virology. Furthermore, your advice always carried a cheerful and encouraging tone. I first emailed you in 2006 and since then you have answered every single email that I have sent to you and considering there were tens of them, many within the email thread titled “one more question”, I thank you for allowing me to draw on your experience and expertise. Without you, our new virus would never have been isolated or identified.

To Cathy Shilton, the greatest ever graduate of the Guelph Zoological medicine-Pathology residency program, thanks for providing me with valuable samples, convincing me of the value of histopathology, and for being so pedantic when reviewing our papers. Cathy, your
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To Jim Wellehan, thank you for your help with the phylogeny of Sunshine virus and the monumental help with our Sunshine virus paper. You are without doubt the most knowledgeable scientist I have ever had anything to do with and I am very grateful to have been allowed to tap into your encyclopaedic knowledge from time to time.

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Abstract

This thesis describes the isolation and molecular identification of a novel paramyxovirus found in Australian snakes. The virus is named Sunshine virus after the geographical origin of the first isolate: the Sunshine Coast of Queensland, Australia. For decades, Australian veterinarians have been presented with snakes displaying neurorespiratory signs. The clinical signs, pathological findings and the results of overseas diagnostic testing, have suggested that at least some of these snakes were infected with paramyxoviruses, most likely ferlaviruses. Australian veterinarians provided 463 samples to be screened for the presence of viruses and nine were found from five snakes from two collections. For all isolates, cytopathic effects consisted of extensive syncytial cell formation with minimal cell lysis. Further characterisation was performed on one of these isolates, BHP1-Lung (from a lung homogenate of a black-headed python [BHP], *Aspidites melanocephalus* with mild respiratory signs and stomatitis). Testing this isolate for haemagglutinating and neuraminidase activity provided equivocal results. The virus could not be found by electron microscopy. Polymerase chain reaction to detect a broad range of paramyxoviruses, reoviruses, adenoviruses and herpesviruses was also non-contributory.

Through the use of high-throughput sequencing, BHP1-Lung was identified as a novel paramyxovirus. Phylogenetic analysis of the nucleoprotein (N), matrix (M), fusion (F) and polymerase (L) proteins clustered this virus within the family *Paramyxoviridae* but outside of both subfamilies. Primers were designed that could detect Sunshine virus which enabled clinical data that is associated with Sunshine virus infection to be described. Clinical signs are typically non-specific (e.g. regurgitation, lethargy, inappetence) and/or can be localised to the neurological and/or respiratory systems. Gross pathology is usually unremarkable. Histopathological findings consist primarily of white matter spongiosis and gliosis of the hindbrain with a mild bronchointerstitial pneumonia.
Conference Proceedings and Publications Arising from this Work

Conference Proceedings

“The Ophidian Paramyxovirus Project at Murdoch University, Perth” Unusual and Exotic Pet (UEP) Special Interest Group (SIG) of the Australian Veterinary Association (AVA) Conference, Te Papa, New Zealand, 2006

“The Ophidian Paramyxovirus Project at Murdoch University – Year 2” Unusual and Exotic Pet (UEP) Special Interest Group (SIG) of the Australian Veterinary Association (AVA) Conference, Cairns, 2007

“The Ophidian Paramyxovirus [OPMV] project at Murdoch University” Australian Veterinary Association Annual Conference, Perth, 2008

“Diagnosing Viral Diseases in Australian Reptiles” Unusual and Exotic Pet (UEP) Special Interest Group (SIG) of the Australian Veterinary Association (AVA) Conference, Alice Springs, 2011

“Diagnosing Viral Diseases in Australian Snakes” College Week of the Australian and New Zealand College of Veterinary Scientists, Gold Coast, 2012

“Sunshine Virus – A Novel Paramyxovirus Found in Australian Pythons” Association of Reptile and Amphibian Veterinarians (ARAV), Oakland, 2012

Papers for Publication


*In this paper the isolation of Sunshine virus into viper heart cells is described. This is followed by the molecular identification of the isolate using high-throughput sequencing and finally, the phylogenetic placement of Sunshine virus is presented.*

Hyndman, T.H., Shilton, C.M., Doneley, R.T. & Nicholls, P.K. *Sunshine virus in Australian Pythons*. *Veterinary Microbiology*, in press

*In this paper the clinical signs, gross pathology and histological findings associated with Sunshine virus infection are described. The results of polymerase chain reaction testing on a variety of clinical samples are also discussed.*


*A review paper of the paramyxoviruses that infect reptiles.*
# List of Abbreviations

*For conciseness, only abbreviations that appear in isolation (i.e. are not always defined) are listed here.*

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AT</td>
<td>adenosine thymidine. An “AT” rich area is one that has a high proportion of adenosine and thymidine residues</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BHP1-Lung</td>
<td>A lung homogenate from a black-headed python with mild respiratory signs and stomatitis. The sample was recovered during an outbreak of neurorespiratory disease in a private collection in Queensland, Australia</td>
</tr>
<tr>
<td>BLASTN</td>
<td>Basic Local Alignment Search Tool for Nucleotides</td>
</tr>
<tr>
<td>BLASTP</td>
<td>Basic Local Alignment Search Tool for Proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BUDR</td>
<td>5’ bromo-3’ deoxyuridine</td>
</tr>
<tr>
<td>BVL</td>
<td>Berrimah Veterinary Laboratories</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>cat. no.</td>
<td>catalogue number</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DBS</td>
<td>dried blood spot</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>an equal mixture of the four deoxynucleotides: deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP)</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded ribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EHV</td>
<td><em>Equine herpes virus</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
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</tbody>
</table>
EMBL European Molecular Biology Library
EMV Encephalomyocarditis virus
EPON a polymer of dodecenyl succinic anhydride hardener 964, Taab 812 resin and methyl nadic anhydride that is used in electron microscopy sample processing
F fusion protein; F0 is the precursor protein and F1 and F2 are the active subunits
FFPE formalin-fixed paraffin-embedded
FDLV Fer-de-Lance virus
g 1. g-force. The g is an abbreviation of gravitational.
   2. gram
G gauge
GSP gene-specific primer
h hour
HA haemagglutination
HAU haemagglutinating units
Hd haemadsorption
HI haemagglutination inhibition
HN haemagglutinin-neuraminidase protein
IgH2 iguana heart cells
IBD inclusion body disease
ICTV International Committee on Taxonomy of Viruses
IHC immunohistochemistry
IgG immunoglobulin G
IgM immunoglobulin M
IgY immunoglobulin Y
IM intra-muscular
ISH in situ hybridisation
JTT Jones-Taylor-Thornton
kDa kilo Daltons
L | 1. RNA-dependent RNA-polymerase of a paramyxovirus. The L is an abbreviation of the word “large”, which refers to the length of this protein  
2. litre  

m | 1. milli  
2. minute  

M | 1. Matrix protein  
2. Molar, moles per litre  

MAFFT | Multiple Alignment with Fast Fourier Transform  

MEM | minimum essential media  

μg | microgram  

μL | microlitre  

μm | micrometre  

µM | micromolar  

mg | milligram  

mL | millilitre  

ML | maximum likelihood  

mm | millimetre  

mM | millimolar  

min | minute  

MW | molecular weight  

N | nucleocapsid protein  

NA | neuraminidase  

NBV | *Nelson Bay* orthoreovirus  

NCDV | *Newcastle disease* virus  

ng | nanograms  

nm | nanometres  

NMWL | nominal molecular weight limit  

NT | Northern Territory, Australia  

OPMV | ophidian paramyxovirus  

ORF | open reading frame
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>P</td>
<td>phosphoprotein</td>
</tr>
<tr>
<td>PAM</td>
<td>point accepted mutation</td>
</tr>
<tr>
<td>PBFDV</td>
<td><em>Psittacine beak and feather disease virus</em></td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>p.i.</td>
<td>post-inoculation</td>
</tr>
<tr>
<td>pmol</td>
<td>picomoles</td>
</tr>
<tr>
<td>PMB</td>
<td>probability matrix from blocks</td>
</tr>
<tr>
<td>PHYLIP</td>
<td>phylogeny interference package</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells, erythrocytes</td>
</tr>
<tr>
<td>RDE</td>
<td>receptor destroying enzyme</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>S</td>
<td>svedbergs</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>sid</td>
<td>once daily. From Latin “<em>semel in die</em>”</td>
</tr>
<tr>
<td>SNT</td>
<td>serum neutralisation test</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infectious dose</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TH-1</td>
<td>terrapene heart cells</td>
</tr>
<tr>
<td>TVMDL</td>
<td>Texas Veterinary Medicine Diagnostic Laboratory</td>
</tr>
<tr>
<td>U</td>
<td>unknown protein</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>V</td>
<td>the cysteine-rich protein V that lies within the phosphoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>VH2</td>
<td>viper heart cells</td>
</tr>
<tr>
<td>VI</td>
<td>virus isolation</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>WAG</td>
<td>Whelan and Goldman</td>
</tr>
</tbody>
</table>
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Acknowledgement of Work Not Performed by the Author

The author acknowledges that Fasteris, a commercial company in Switzerland, carried out a series of sample preparation steps before performing high-throughput sequencing on RNA extracted from viper heart cells infected with BHP1-Lung (= Sunshine virus). The phylogenetic work on Sunshine virus was performed by Dr James Wellehan. The gross pathology and histopathological findings associated with Sunshine virus infection were described by Dr Cathy Shilton. The reader will be reminded of these contributions in the relevant sections of this thesis. The rest of the work presented here was performed by the author.
Chapter 1 - Purpose of Project and Outline of Thesis

1.1 Purpose of Project

This thesis defines an ongoing problem seen in Australian snakes and discusses the methods and results of an investigation into this problem. Australian snakes with neurorespiratory signs of disease have, for many years, been presented to veterinarians. Outbreaks of these signs involving multiple animals have been seen in both private and public Australian snake collections. In some instances, veterinarians have pursued overseas testing to look for evidence of a paramyxoviral infection. Many of these affected snakes do not respond to supportive treatment and some die naturally or are euthanased. The clinical signs and pathological findings, and the results of overseas diagnostic testing, have all been consistent with an infection by a paramyxovirus. However, these findings should not be viewed as incontrovertible. There are no peer-reviewed published reports that definitively confirm a diagnosis of a viral infection in any of these snakes.

The broad aims of this project are therefore:

1. To isolate and identify a virus from Australian snakes that could explain the clinical signs, pathological findings and overseas diagnostic test results that have been suggestive but not conclusive of a paramyxovirus infection.

2. To investigate if the association between the presence of this isolated virus and disease is causative.

3. To produce a diagnostic test that could rapidly screen Australian snakes for the presence of this virus.

4. To apply this diagnostic test to Australian snake samples to provide clinically useful information about the biology of this viral infection e.g. shedding patterns, tissue predilections.
1.2 Outline of Thesis

This thesis describes an investigation of Australian snakes that resulted in the isolation and description of a novel paramyxovirus. In Figure 1.1, a chapter-by-chapter overview of this thesis is presented. This chapter provides an overview of the structure and content of each of the next seven chapters. Chapter two provides a critical review of the current and published literature that is relevant to the paramyxoviruses of snakes. This will equip the reader with the relevant information that is necessary to understand the methods that were subsequently utilised to screen Australian snake samples for the presence of a range of viruses, particularly paramyxoviruses. In chapter three, unpublished data in the form of clinical signs, histopathology reports and the results of overseas diagnostic testing are presented and critiqued. This chapter identifies the limitations of this data and provides the justification for a formal investigation of snakes affected with neurorespiratory signs, or other signs, considered consistent with a viral infection.

Chapter four describes the suite of methods that were used to investigate Australian snake samples for the presence of viruses, mainly paramyxoviruses. This chapter also includes brief experiments that were performed on positive control material that aimed to optimise or adapt these methods to be more suitable to the samples that would be received.

Chapter five describes the results of testing 463 Australian snake samples that were collected from animals suspected of harbouring a paramyxovirus. The testing involved screening samples for the presence of haemagglutinating agents and neuraminidase activity. Blood samples were screened for the presence of anti-ferlaviral antibodies. Virus isolation and polymerase chain reaction were performed on swab and tissue samples. Nine viruses were eventually isolated from five snakes and, following this, further testing was performed on one of these isolates (BHP1-Lung) in an attempt to determine the family it belongs to.
Chapter six continues the investigation to determine the identity of this isolate. Analysis of the data produced from high-throughput sequencing facilitated the molecular identification of BHP1-Lung as a paramyxovirus. Partial characterisation of the six major open reading frames and phylogenetic analysis is described and revealed that this paramyxovirus was unique and did not cluster with either existing paramyxoviral subfamily. This novel paramyxovirus was named Sunshine virus after the geographical origin of the first isolate: the Sunshine Coast of Queensland, Australia. A set of polymerase chain reaction (PCR) primers was then designed to rapidly detect this virus in a range of clinical samples.

Chapter seven describes the results of testing 141 samples with this newly-designed PCR. Animals infected with Sunshine virus could be identified and this allowed the clinical signs and histopathological findings that were associated with infection to be described. Finally, chapter eight provides a summarising discussion of the investigations performed. This final chapter provides a number of directions that future research on Sunshine virus can follow.

For those reading this thesis electronically, the Table of Contents, List of Figures, List of Tables, cross-references in bold type, and in-text citations have been inserted as hyperlinks. This allows the reader to press the Ctrl (Control) key on their keyboard while left-clicking their mouse on the hyperlink to be taken directly to the relevant section of the thesis or bibliography.
Figure 1.1 Overview of the eight chapters presented in this thesis.
Chapter 2 – Literature Review

2.1 Introduction

A wide range of pathogenic viruses have been detected in snakes throughout the world and the interested reader is referred to the comprehensive reviews by Wellehan and Johnson (2005), Jacobson (2007), Marschang (2011) and Ariel (2011). Paramyxoviruses have been detected in a diverse range of host species and have often been associated with significant morbidity and mortality (Jacobson, 2007). This literature review will provide background information on the paramyxoviruses, concentrating on those that infect snakes (2.1 – Introduction); the clinical disease that they are capable of producing in snakes (2.2 – The Disease) and lastly; the specific laboratory tests that are available to detect, or suggest, the presence of these viruses in live and dead snakes (2.3 – Diagnostic Tests).

2.1.1 General Information on Paramyxoviruses

Members of the family Paramyxoviridae are currently divided into two subfamilies: Pneumovirinae and Paramyxovirinae (Lamb et al., 2005). The subfamily Pneumovirinae includes the two genera Pneumovirus (e.g. Human respiratory syncytial virus) and Metapneumovirus (e.g. Avian metapneumovirus), while the subfamily Paramyxovirinae currently contains seven genera: Respirovirus (e.g. Sendai virus), Morbillivirus (e.g. Measles virus), Rubulavirus (e.g. Mumps virus), Henipavirus (Hendra virus and Nipah virus), Avulavirus (e.g. Newcastle disease virus), Aquaparamyxovirus (Atlantic salmon paramyxovirus) and Ferlavirus (Fer-de-lance paramyxovirus) (ICTV, 2012).

Paramyxoviruses are important viruses of animals. Newcastle disease virus, Nipah virus, Peste des Petits Ruminants virus, Rinderpest virus and Turkey rhinotracheitis virus are all paramyxoviruses that cause OIE (Office International des Epizooties)-Listed Diseases (OIE, 2011). These five paramyxoviruses are joined by two more, Menangle virus and Hendra virus,
and these seven viruses all feature on the Australian Government’s National Notifiable Animal Diseases List (Australia, 2011).

All paramyxoviruses share a common structure consisting of a spiked envelope surrounding a negative sense, single-stranded, RNA genome, and various other functional proteins (Lamb and Parks, 2007). Paramyxoviruses are often spherical but can be filamentous to pleomorphic (Lamb and Parks, 2007). Sizes typically range from 150-350nm (Lamb and Parks, 2007). Figure 2.1 illustrates the general appearance of a paramyxovirus.

**Figure 2.1** All images from Lamb and Parks (2007) **Left:** Schematic representation of a typical paramyxovirus. RNP = RNA nucleocapsid phosphoprotein. **Top right:** Negative stain, transmission electron microscopic (TEM) appearance of *Parainfluenza virus* type 5, a typical paramyxovirus showing a spiked envelope (226,280 X). **Bottom right:** Negative stain, TEM; disrupted Sendai viruses showing typical “herring-bone” nucleocapsid proteins. Bar represents 100nm. The unique structure of the nucleocapsid is a key diagnostic feature of *Paramyxoviridae*.

A typical and simplified life cycle of a paramyxovirus has been described by Lamb and Parks (2007). The major antigenic determinant of a paramyxovirus is its haemagglutinin-neuraminidase (HN) protein which is responsible for attachment to the host cell’s plasma membrane and the *in vitro* haemagglutination of certain vertebrate erythrocytes. Once attached, the viral fusion protein (F) facilitates entry into the host cell by fusing the viral envelope with the cell membrane of the host. The RNA genome of the virus is then transferred to the cytoplasm of the host cell where the viral RNA-dependent RNA polymerase (L, large) is
used to replicate the virus’s genome and to create viral transcripts which code for, and initiate the translation of the viral proteins. The nucleocapsid (N) protein that is produced encapsulates the viral RNA, protecting it from nuclease digestion. The phosphoprotein (P) and cysteine-rich protein V (V) are the two proteins that can be synthesised by the polycistronic P/V gene. The phosphoprotein is a component of the polymerase complex, while the cysteine-rich protein V (V) can be expressed to attempt to avoid host interferon defences. The matrix (M) protein is the most abundant structural protein and assembles between the envelope and nucleocapsid core. It provides an anchor point for the F and HN proteins. Once the viral genome and its proteins have been assembled, they are released from the host cell through either cell lysis or by budding from the membrane. The viral envelope is formed from the plasma membrane of the host cell.

More detailed reviews about paramyxoviral genomes, including leader sequences and intergenic regions, have been published elsewhere (Lamb et al., 2005; Lamb and Parks, 2007).

2.1.2 Ophidian Paramyxovirus or Ferlavirus

The literature has not shown conformity in how it refers to snake paramyxoviruses. Since the first characterisation of a snake paramyxovirus that was named Fer de Lance Virus (FDLV) (Clark et al., 1979), the term ophidian paramyxovirus (often abbreviated to OPMV or oPMV) has also been used to describe the paramyxoviruses found in snakes (Lloyd and Flanagan, 1991; Homer et al., 1995; Jacobson et al., 1997; Manvell et al., 2000; Kindermann et al., 2001; Oros et al., 2001; Nogueira et al., 2002; Kolesnikovas et al., 2006). In 2009, a proposal was put forward by Kurath (2009) to the International Committee on Taxonomy of Viruses (ICTV) to create the new genus Ferlavirus with Fer-de-lance paramyxovirus (the same virus as FDLV) as its type species. Prior to the work presented in this thesis, all reptilian paramyxoviruses had clustered together within the Ferlavirus genus (Marschang et al., 2009). The proposal by Kurath has since been accepted by the ICTV and so Ferlavirus appears, for the first time, in the 2011 release of Virus Taxonomy (ICTV, 2012). This thesis will therefore refer to the viruses of
this proposed genus as “ferlaviruses”. The term “ophidian paramyxovirus” or “OPMV” will only be used where the relevant references have specifically referred to this virus as such and where not doing so would create confusion. In this thesis, the discovery of a new paramyxovirus of snakes, that is distantly related to the ferlaviruses, is described, and it is proposed that this new virus be named Sunshine virus after the geographical origin of the first isolate, the Sunshine Coast of Queensland. Therefore, the term “snake paramyxovirus” will encompass both the ferlaviruses that occur in snakes, and Sunshine virus. The term “reptilian paramyxovirus” will refer to all paramyxoviruses that have been reported in any reptile.

2.1.3 Specific Information about Ferlaviruses

In 1972, an outbreak of neurorespiratory disease in a Swiss serpentarium formed the basis for the first description of a paramyxovirus isolated from a snake (Folsch and Leloup, 1976). The physicochemical traits of this first isolate were then characterised and described by Clark et al. (1979). A personal communication mentioned by Kolesnikovas et al. (2006) states that the Brazilian lancehead vipers (Bothrops moojeni but incorrectly referred to as Fer-de-Lance vipers [B. atrox] in earlier works) in the Swiss serpentarium originated from Brazil. No further information is provided.

The report by Folsch and Leloup (1976) may not have been the first recorded outbreak of a paramyxovirus in a collection of snakes. Lunger and Clark (1978) describe a 1970 study about an over-crowded collection of Japanese shim-hebi snakes (Elaphe quadrivirgata) that were being housed in a restaurant. A high mortality rate, clinical signs arising from the respiratory tract (respiratory distress, mucus secretions) and histopathological findings bearing a similarity to mammalian alveolitis were seen. Lunger and Clark (1978) speculate that this outbreak may have been caused by a viral pneumonia. However, even if this outbreak was proven to have a viral aetiology, it may not have been a paramyxovirus.
The entire genome of *Fer-de-lance paramyxovirus* (referred to as Fer-de-Lance Virus [FDLV] in this report) has been determined (Kurath et al., 2004). The genome was 15,378 nucleotides long and was made up of seven distinct genes: 3’ – Nucleocapsid (N) – Unknown (U) – Phosphoprotein/Protein V (P/V) – Matrix (M) – Fusion (F) – Haemagglutinin-Neuraminidase (HN) – RNA-Dependent RNA Polymerase (L). Six of these are common to most paramyxoviruses (section 2.1.1 – General Information on Paramyxoviruses) but a seventh, and novel, gene was also reported. This gene was designated (U) for “unknown” as its function was unidentified.

Since this study, Marschang (2009) has found evidence of this gene in several more ferlaviral isolates, including isolates from lizard and chelonian hosts. Interestingly, a study published eight years before Kurath et al. (2004) described the electrophoretic separation of the radiolabelled proteins of ferlavirus-infected and uninfected cell culture (Richter et al., 1996). The six major proteins common to most paramyxoviruses were identified but “other less abundant proteins also were seen in infected cells and may represent truncated forms of structural proteins”. It is likely that one of these “other less abundant proteins” may have actually been encoded by the (U) gene that seems to be unique to the ferlaviruses. However, because these proteins were not characterised and because the Richter et al. (1996) and Kurath et al. (2004) studies investigated different ferlaviral isolates, it still remains possible that the (U) protein may only be found in some isolates of ferlavirus.

In 2006, Franke et al. reviewed the paramyxoviral fusion protein and then provided a description of the sequence motifs of this protein in a ferlavirus. The fusion protein is thought to be made up of a precursor protein, named F0, that is cleaved by host cell enzymes into its active subunits: F1 and F2. In the case of the highly virulent (velogenic) strains of *Newcastle disease virus* (NCDV), a host cell protease named Furin cleaves the viral F0 protein into its subunits. Furin is found within the Golgi apparatus of a wide range of tissues and organs and this is believed to explain why infections with velogenic strains of NCDV involve multiple organs. Franke et al. (2006) demonstrated that ferlaviruses have a fusion protein that is
cleaved by Furin. This may help explain the observations that ferlaviruses can cause severe illness (Folsch and Leloup, 1976; Jacobson et al., 1992) and be isolated (Clark et al., 1979), immunohistochemically detected (Homer et al., 1995) and detected by polymerase chain reaction (PCR) (Papp et al., 2010a), in a wide range of snake organs.

The various isolates of ferlavirus have been assembled into phylogenetic trees to characterise the diversity within this proposed genus (Ahne et al., 1999b; Franke et al., 2001; Kindermann et al., 2001; Marschang et al., 2009; Papp et al., 2010a; Papp et al., 2010b; Abbas et al., 2011) (Figure 2.2 left). Results indicate that the isolates of ferlavirus can be placed into three groups (Ahne et al., 1999b): groups a and b form distinct groups, and it has been suggested that these form two distinct species, while the third group, i, is made up of various intermediate isolates. Broader phylogenetic comparisons between a ferlavirus and other paramyxoviral genera have also been reported (Junqueira de Azevedo et al., 2001; Kurath et al., 2004; Marschang et al., 2009) (Figure 2.2 right). Junqueira de Azevedo et al. (2001) analysed a region of the F gene and found it was distantly related, although still most homologous, to Sendai virus and Human parainfluenza virus types 1 and 3.

The lack of serological relatedness of ferlaviruses to other paramyxoviruses has been reported by several authors (Clark *et al.*, 1979; Potgieter *et al.*, 1987; Blahak, 1995; Richter *et al.*, 1996; Ahne *et al.*, 1999b). Clark *et al.* (1979) titrated the antisera of 16 paramyxoviruses and 3 orthomyxoviruses against a ferlavirus and then did the reverse by titrating ferlavirus antisera against these same 19 myxoviruses. It was concluded that ferlavirus was antigenically distinct from other myxoviruses. Like Clark *et al.* (1979), Richter *et al.* (1996) showed that the antisera specific for *Parainfluenza virus* types 1, 2 and 3 (presumed to be *Human parainfluenza virus*), *Newcastle disease virus*, *Respiratory syncytial virus* (presumed to be *Human respiratory syncytial virus*) or *Canine distemper virus* did not inhibit the haemagglutinating ability of three ferlaval isolates. Ahne *et al.* (1999b) was also unable to demonstrate any cross-reactivity between ferlaval antisera and a range of paramyxoviruses: *Mumps virus*, *Measles virus*, *Sendai virus* and *Newcastle disease virus*. 
In contrast to these findings, serological relationships between ferlavirus and other myxoviruses have been shown by other authors. Blahak (1995) demonstrated a serological relationship between ferlavirus and *Avian paramyxovirus* types 1 and 7 prompting Manvell *et al.* (2000) to classify two isolates of ferlavirus as “ophidian paramyxovirus type 1 (PMV-1) and ophidian paramyxovirus type 7 (PMV-7)”. In another report, Potgieter *et al.* (1987) used immunohistochemical staining to detect ferlavirus in a section of infected snake lung after the lung had been treated with the fluorescently-labelled antisera of *Parainfluenza virus* type 2.

It is possible that the seemingly contradictory results about the serological relatedness of ferlaviruses to other paramyxoviruses are actually all valid and not contradictory. The disparity could be explained if the various ferlaviral isolates showed diversity in their serological relatedness to each other (Lloyd *et al.*, 2005). If this were the case, then certain isolates of ferlavirus could show serological relatedness to some paramyxoviruses where others did not. But when all the findings in the literature are considered, it seems reasonable to conclude that the serological relatedness of the ferlaviruses to other paramyxoviruses is, at best, limited.

The next two sections concern themselves with ferlaviral disease (*2.2 – The Disease*) and the tests that have been used to diagnose ferlaviral infection (*2.3 – Diagnostic Tests*).

### 2.2 The Disease

#### 2.2.1 Clinical Signs and Differential Diagnoses

Ferlaviral infections have been associated with highly pathogenic disease outbreaks (Folsch and Leloup, 1976; Jacobson *et al.*, 1980b; Jacobson *et al.*, 1981; Jacobson *et al.*, 1992; Kolesnikovas *et al.*, 2006). Infection has been detected in several snake families: Colubridae, Elapidae, Viperidae, Crotalidae, Boidae\(^1\) and Pythonidae (Jacobson *et al.*, 1997; Ahne *et al.*, 1999b; Oros *et al.*, 2001). One report described the clinical signs associated with ferlaviral

\(^1\) It has not always been clear whether pythons are a subfamily (Pythoninae) of Boidae or are a family in their own right. However, recent molecular data suggest that pythons should form their own family, Pythonidae (Dong and Kumazawa, 2005; Noonan and Chippindale, 2006).

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infection as being variable, often non-specific, and occasionally subtle (Sand et al., 2004). When clinical signs can be attributed to a particular organ system, they are most commonly localised to the respiratory tract (Jacobson, 2007) but there are also reports about snakes suffering from neurological disease (Figure 2.3). Table 2.1 outlines the clinical signs reported by various authors.
Figure 2.3 Burmese python (Python molurus bivittatus; top) (Bronson and Cranfield, 2006) and rock rattlesnake (Crotalus lepidus; middle) (Jacobson, 2007) showing abnormal posturing and an inability to right. Bottom: Aruba Island rattlesnake (Crotalus unicolor) with a haemorrhagic oral cavity (Jacobson, 2007).
<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-specific</strong></td>
<td></td>
</tr>
<tr>
<td>Anorexia</td>
<td>(Jacobson et al., 1992; Manvell et al., 2000; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Regurgitation (occasional)</td>
<td>(Jacobson et al., 1981; Jacobson et al., 1992; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Mucoid diarrhoea or malodorous stools</td>
<td>(Jacobson et al., 1981; Jacobson et al., 1992; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Lethargy/Moribund</td>
<td>(Folsch and Leloup, 1976; Ahne et al., 1987a)</td>
</tr>
<tr>
<td>Sudden death</td>
<td>(Jacobson et al., 1981; Jacobson et al., 1997; Marschang et al., 2009; Papp et al., 2010a)</td>
</tr>
<tr>
<td><strong>Respiratory</strong></td>
<td></td>
</tr>
<tr>
<td>Not seen</td>
<td>(Jacobson et al., 1992; West et al., 2001)</td>
</tr>
<tr>
<td>Not described further</td>
<td>(Kolesnikovas et al., 2006; Marschang et al., 2009)</td>
</tr>
<tr>
<td>Brown to haemorrhagic discharge from nostrils and/or trachea or in oral cavity</td>
<td>(Jacobson et al., 1981; Jacobson et al., 1997)</td>
</tr>
<tr>
<td>Stridor and/or respiratory noise</td>
<td>(Manvell et al., 2000)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>(Nogueira et al., 2002; Papp et al., 2010a)</td>
</tr>
<tr>
<td>Clear mucus in mouth</td>
<td>(Potgieter et al., 1987)</td>
</tr>
<tr>
<td>Clear nasal discharge</td>
<td>(Manvell et al., 2000)</td>
</tr>
<tr>
<td>Mouth gaping</td>
<td>(Folsch and Leloup, 1976; Jacobson et al., 1981)</td>
</tr>
<tr>
<td><strong>Neurological</strong></td>
<td></td>
</tr>
<tr>
<td>Not described further</td>
<td>(Papp et al., 2010a)</td>
</tr>
<tr>
<td>Complete flaccid paralysis</td>
<td>(West et al., 2001)</td>
</tr>
<tr>
<td>Decreased cutaneous sensation</td>
<td>(West et al., 2001)</td>
</tr>
<tr>
<td>Head tremors</td>
<td>(Jacobson et al., 1980b; Jacobson et al., 1992; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Abnormal posturing/disequilibrium i.e. opisthotonus (star gazing) or inability to right itself</td>
<td>(Folsch and Leloup, 1976; Jacobson et al., 1980b; Jacobson et al., 1992; Kolesnikovas et al., 2006; Papp et al., 2010a)</td>
</tr>
</tbody>
</table>

Table 2.1 Clinical signs of ferlaviral infection described in the literature. Only the references that report on original data are included here.
In 1991, Lloyd and Flanagan described the clinical manifestations of ferlaviral infection as fitting into three discrete clinical syndromes. Firstly, there are snakes affected acutely or peracutely who usually show no premonitory signs except for possibly respiratory and/or neurological compromise. The time from exposure to clinical disease can be six to ten (or more) weeks. The second category involves the “poor doers”. Anorexia, hypophagia and regurgitation are often the first signs seen. It may be up to seven months before other signs are seen that may include reluctance to move, increased use of heat pads, emaciation and poor muscle tone. These snakes often have high anti-ferlaviral antibody titres by haemagglutination inhibition but viral shedding proceeds. The last group identifies the clinically healthy animals that shed virus in the face of high antibody titres. These snakes may be carriers and be asymptomatic for up to ten months but eventually become chronic “poor doers”. These observations were based on clinical experience and were not from a controlled study.

The clinical signs attributed to ferlaviral infection are variable and often non-specific. A long list of disease processes is associated with non-specific clinical signs but a shorter list exists for snakes with respiratory and/or neurological signs of disease (Table 2.2). Anamnesis and physical examination will exclude many of these diseases leaving only a few diagnoses that include respiratory and neurological disease. In these cases, ferlavirus infection should remain in the clinician’s mind until compelling evidence suggests otherwise.
Primary Differential Diagnoses

Respiratory
Viral: Inclusion body disease (IBD), reovirus
Bacterial: *Mycoplasma* sp., *Chlamydophila* sp., *Pasteurella* sp.,
Fungal: *Mucor* sp., *Penicillium* sp., *Aspergillus* sp., *Candida* sp.
Parasitic: nematodes (e.g. lungworm), pentastomids, trematodes
Infectious periodontal disease
Nutritional: hypovitaminosis A, obesity
Neoplasia
Aspiration pneumonia
Poor husbandry: too hot, topical irritants (e.g. disinfectant fumes)
Ascites

Neurological
Viral: Inclusion body disease (IBD), reovirus, adenovirus
Parasitic: visceral larval migrans (aberrant ascarid migration), protozoal
Nutritional: hypovitaminosis B1, biotin deficiency, hypocalcaemia, ketoacidosis, hypoglycaemia
Drug toxicities: ivermectin, insecticide
Hepatoencephalopathy
Renomegaly
Head and/or spinal trauma
Vestibular disease

| Table 2.2 | Differential diagnoses for respiratory and neurological disease in snakes. Adapted from Girling and Raiti (2004) and Murray (2006). Secondary and tertiary causes of neurological and respiratory disease (e.g. *Pseudomonas* sp. pneumonia) have been excluded for conciseness. |

There is only limited information available describing the signalments that have increased predilections for ferlaviral infection. During an outbreak of ferlaviral infection in a zoological collection of 438 snakes, all the snakes that died (35) were adults and both sexes were affected equally (Jacobson *et al*., 1981). The exact age of most of the 438 snakes in this collection was not known and the ages that were known were not presented in this report, so the average age at death could not be determined. The unsupported claim has been made that geriatric and young snakes are more likely to succumb to this disease (Ritchie, 2006).
Ritchie (2006) states that stress, shipping, temperature fluctuations and overcrowding may exacerbate subclinical infections. Ritchie (2006) also suggests that strain variation may dictate host specificity and similarly in cases where non-host adapted strains occur, wider outbreaks may be anticipated. In a more recent report, Papp et al. (2010a) investigated the strains of ferlavirus involved in disease outbreaks. Not only was it shown that several distinct strains could be found in an outbreak in a single collection but multiple distinct strains could also be found in a single snake.

### 2.2.2 Gross Pathology

Considering the clinical signs that are associated with ferlaviral infection, it is not surprising that any significant changes that are seen at necropsy are often localised to the respiratory system (Table 2.3 and Figure 2.4). It is important to note that more than one author has not detected any gross post mortem changes in snakes that were later identified to be infected with ferlavirus.

<table>
<thead>
<tr>
<th>Gross Pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(Lloyd and Flanagan, 1991; Jacobson et al., 1997; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Pulmonary congestion or oedema</td>
<td>(Folsch and Leloup, 1976; Potgieter et al., 1987; Oros et al., 2001; Kolesnikovas et al., 2006; Jacobson, 2007)</td>
</tr>
<tr>
<td>Haemorrhagic pneumonia</td>
<td>(Jacobson et al., 1992; Jacobson et al., 1997; Oros et al., 2001; West et al., 2001)</td>
</tr>
<tr>
<td>Blood in oral cavity or free in coelom</td>
<td>(Jacobson et al., 1997; Jacobson, 2007)</td>
</tr>
<tr>
<td>White nodules on liver</td>
<td>(Jacobson et al., 1992)</td>
</tr>
<tr>
<td>Mucoid or caseous exudate in the lung</td>
<td>(Jacobson et al., 1980b; Jacobson et al., 1981)</td>
</tr>
<tr>
<td>Diffuse to focal accumulations of caseous necrotic debris in pulmonary tissue</td>
<td>(Jacobson et al., 1992; Oros et al., 2001)</td>
</tr>
</tbody>
</table>

**Table 2.3** Gross pathological changes associated with ferlaviral infection
Figure 2.4 Top: Severe haemorrhage in a lung from an Eastern diamondback rattlesnake (Crotalus adamenteus) that was infected with ferlavirus (Jacobson, 2007). Bottom: Cut lung showing thickening and a frothy discharge (Bronson and Cranfield, 2006).

It has been suggested by Lloyd and Flanagan (1991) that in the event of a snake’s death during a suspected outbreak of ferlavirus, samples should be collected in triplicate. One sample to be collected into formalin for histopathological assessment; a second to be collected into formalin for 48 hours and then transferred to 70% ethanol, which will facilitate any special staining that is required; and lastly, samples should be frozen down for virus isolation. Blood should also be taken from both in-contact and adjacent animals for anti-ferlavirus antibody detection.
Although not mentioned in this report, tissue samples can also be used for the detection of nucleic acids by polymerase chain reaction and \textit{in situ} hybridisation.

\textbf{2.2.3 Histopathology}

There are no histological signs that are pathognomonic for ferlaviral infection (Ritchie, 2006). Instead, a wide range of histopathological findings have been reported that are most commonly attributed to the respiratory and neurological systems (Table 2.4). Intranuclear or intracytoplasmic viral inclusions may be seen and these should heighten the pathologist’s suspicions of ferlaviral infection (Jacobson, 2007). Ultrastructurally, these inclusions have been shown to consist of strands of viral nucleocapsid (Jacobson, 2007). Although less specific for ferlaviral infection than viral inclusions, proliferative pneumonia (Figure 2.5) and perivascular cuffing in the brain (Figure 2.6) are the changes most commonly reported in the literature. Jacobson \textit{et al.} (2001a) has noted that inclusion body disease (IBD), mycoplasmosis and infection with reovirus form important rule-outs during an investigation of snakes affected with proliferative pneumonia.

Through the use of immunohistochemistry, Homer \textit{et al.} (1995) was able to localise pulmonary infections to the luminal surface and cytoplasm of faveolar epithelium. With a similar purpose, Sand \textit{et al.} (2004) used \textit{in situ} hybridisation to locate ferlavirus in a variety of infected organs. Virus was intranuclear in the brain while being intracytoplasmic in hepatocytes, Kupffer cells, pulmonary alveolar [faveolar] macrophages, respiratory epithelial cells and renal tubular epithelial cells.
<table>
<thead>
<tr>
<th>Histopathological Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respiratory</strong></td>
<td></td>
</tr>
<tr>
<td>Moderate to diffuse amounts of cellular debris and exudate filling airways</td>
<td>(Jacobson et al., 1980b; Jacobson et al., 1981)</td>
</tr>
<tr>
<td>Varying amounts of mixed inflammatory cells in the interstitium</td>
<td>(Jacobson et al., 1980b; Jacobson et al., 1981; Potgieter et al., 1987;</td>
</tr>
<tr>
<td>Jacobson et al., 1992; Jacobson et al., 1997; Oros et al., 2001; West et al., 2001;</td>
<td></td>
</tr>
<tr>
<td>Kolesnikovas et al., 2006; Jacobson, 2007)</td>
<td></td>
</tr>
<tr>
<td>Gram negative micro-organisms seen</td>
<td>(Homer et al., 1995; Oros et al., 2001; Jacobson, 2007)</td>
</tr>
<tr>
<td>Hyperplastic alveolar [faveolar] cells</td>
<td>(Homer et al., 1995; Jacobson et al., 1997; Oros et al., 2001; Jacobson,</td>
</tr>
<tr>
<td>Jacobson et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>Thickened pulmonary septae</td>
<td>(Homer et al., 1995; Jacobson et al., 1997; Jacobson, 2007)</td>
</tr>
<tr>
<td>Hyperplasia and often hypertrophied epithelium</td>
<td>(Jacobson et al., 1981; Homer et al., 1995)</td>
</tr>
<tr>
<td>Small numbers of pale eosinophilic intracytoplasmic (or not described) inclusions</td>
<td>(Jacobson et al., 1981; Potgieter et al., 1987; Homer et al., 1995;</td>
</tr>
<tr>
<td>Jacobson et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>Giant cell formation</td>
<td>(Homer et al., 1995; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Lesion severity decreases from cranial to middle to caudal lung area*</td>
<td>(Jacobson et al., 1997)</td>
</tr>
<tr>
<td><strong>Neurological</strong></td>
<td></td>
</tr>
<tr>
<td>Eosinophilic intracytoplasmic inclusion bodies</td>
<td>(West et al., 2001)</td>
</tr>
<tr>
<td>Demyelination and degeneration of axon fibres</td>
<td>(Jacobson et al., 1980b)</td>
</tr>
<tr>
<td>Lymphohistiocytic neuritis of oesophagus</td>
<td>(West et al., 2001)</td>
</tr>
<tr>
<td>Multifocal neuronal degeneration</td>
<td>(West et al., 2001)</td>
</tr>
<tr>
<td>Moderate axonal sheath ballooning</td>
<td>(Jacobson et al., 1980b)</td>
</tr>
<tr>
<td>Multifocal gliosis</td>
<td>(Jacobson et al., 1980b)</td>
</tr>
<tr>
<td>Perivascular cuffing in the brain</td>
<td>(Jacobson et al., 1980b; West et al., 2001; Jacobson, 2007)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>Pancreatitis and/or pancreatic necrosis and/or pancreatic fibrosis</td>
<td>(Jacobson et al., 1980b; Jacobson et al., 1992; Kolesnikovas et al., 2006;</td>
</tr>
<tr>
<td>Jacobson, 2007)</td>
<td></td>
</tr>
<tr>
<td>Pancreatitisic giant cell formation</td>
<td>(Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Pyogranulomatous hepatitis</td>
<td>(Jacobson et al., 1992)</td>
</tr>
<tr>
<td>Gram negative infections seen in many organs</td>
<td>(Jacobson et al., 1992)</td>
</tr>
</tbody>
</table>

*These findings were in snakes that were experimentally infected with ferlavirus by endotracheal inoculation. 
*Brain was not examined histologically in Jacobson et al. (1981), Potgieter et al. (1987), Jacobson et al. (1992), Homer et al. (1995) or Jacobson et al. (1997). It is unclear whether the brain was examined in Oros et al. (2001) and Kolesnikovas et al. (2006).
Figure 2.5 All images from Jacobson (2007). **Top:** Normal lung of a bush viper (*Atheris squamiger*). SM = smooth muscle bundle. FV = faveolae (airways). **Middle:** Normal lung of a western diamondback rattlesnake (*Crotalus atrox*). SP = connective tissue septae. **Bottom:** Aruba Island rattlesnake (*Crotalus unicolor*) experimentally infected with ferlavirus. There are large amounts of debris and inflammatory cells in the airways. Thickened pulmonary septae are also noted.
2.2.4 Non-Ferlaviral Microbiological Findings

A number of authors have reported on the non-viral microbiological findings in snakes infected with ferlavirus. Gram negative bacterial pathogens have been cultured from various organs: *Aeromonas, Citrobacter, Escherichia, Enterobacter, Morganella, Proteus, Providencia, Pseudomonas, Salmonella* and *Serratia* (Folsch and Leloup, 1976; Jacobson *et al.*, 1981; Jacobson *et al.*, 1992; Homer *et al.*, 1995; Oros *et al.*, 2001; Kolesnikovas *et al.*, 2006; Jacobson, 2007). In one of these studies, fungal elements could not be cultured (Kolesnikovas *et al.*, 2006). It has been suggested that ferlaviral infections may be immunosuppressive, possibly due to lymphoid depletion (Oros *et al.*, 2001), allowing secondary bacterial invaders (Kolesnikovas *et al.*, 2006). So while it is recommended that concurrent complicating bacterial infections be treated as early as possible (Jacobson *et al.*, 1992; Kolesnikovas *et al.*, 2006), antibiotic use may not provide any improvement in already affected snakes (Folsch and Leloup, 1976; Jacobson *et al.*, 1981).
Papp et al. (2010a) has described the isolation of reoviruses from various organs from four snakes that were positive by PCR for the presence of ferlavirus. In a second study, Abbas et al. (2011) simultaneously detected at least one atadenovirus, a reovirus and a ferlavirus in each of three corn snakes. One of these snakes, a juvenile, was vomiting and displayed dyspnoea before dying. These recent studies form the first reports of mixed viral infections in snakes infected with ferlavirus.

2.2.5 Transmission

Little is known about ferlaviral transmission. Koch’s postulates were fulfilled after the successful infection and re-isolation of ferlavirus in a group of snakes (Jacobson et al., 1997). Ferlavirus was obtained from cell culture supernatant and then inoculated into the trachea of six naïve captive-bred Aruba Island rattlesnakes (Crotalus unicolor). Prior to viral challenge, anti-ferlaviral antibodies could not be detected by haemagglutination inhibition in any of these snakes. No further pre-infection testing was done, however, rapid detection using polymerase chain reaction did not become routine until effective primers were later published (Ahne et al., 1999b). Once challenged, three snakes were euthanased at 4, 8 and 15 days post-inoculation (p.i.), respectively, while another two died at 19 and 22 days p.i. The sixth snake served as a control and was euthanased at day zero without viral inoculation. Another three snakes were sham-inoculated with uninfected cell culture supernatant. Ferlavirus was successfully isolated from the lungs of all the snakes that had been inoculated with virus and none of the sham-inoculated snakes thus Koch’s postulates were satisfied.

The incubation period of ferlavirus in naturally-acquired infections is unknown. It is problematic to deduce the incubation period from the aforementioned study by Jacobson (1997) because this study experimentally inoculated a small number of animals (n=6) from one species with an amount of virus that is difficult to compare to a naturally-acquired infection. There are unsupported claims in the literature that the incubation period for ferlavirus may be
as short as 21 days (Hernandez-Divers, 2006) but will generally exceed 90 days (Hernandez-Divers, 2006; Ritchie, 2006).

Pasmans et al. (2007) states that ferlavirus is easily transmitted through both aerosols and contact and terraria for individually housed snakes provide little defence against the transmission of ferlavirus. Hernandez-Divers (2006) makes the unsupported claim that it can be transmitted from snake to snake by direct contact, respiratory secretions, fomites and ectoparasites (especially mites). Considering that ferlavirus has been isolated from the sputum of a rattlesnake (Crotalus durissus terrificus) (Nogueira et al., 2002) and detected by polymerase chain reaction (PCR) in both oral and cloacal swabs (Rachel Marschang, personal communication), it is reasonable to assume that ferlavirus can be transmitted between snakes by both oral secretions and cloacal excretions. To the best of the author’s knowledge, ferlavirus has not been isolated, or detected by PCR, from fomites or ophidian ectoparasites.

Jacobson et al. (1980b) describes an outbreak of ferlavirus after a newly-introduced rock rattlesnake (Crotalus lepidus) was placed into direct contact with eight snakes of the same species. All nine of these snakes died but several colubrids kept in the same room were unaffected. It is possible that (1) this strain of ferlavirus did not affect the colubrids because it was not transmitted to the snakes that were not in direct contact; (2) that the colubrids were infected without being overtly affected; or (3) that this strain of ferlavirus was not infectious to colubrids. In an outbreak of ferlavirus in a collection of 446 snakes that were housed in three rooms, 87% of the snakes in one room died, 40% of the second room occupants died and all the snakes in the third room were unaffected (Folsch and Leloup, 1976). In the first two rooms, the snakes that were in the closest proximity to the rooms’ doors had the highest mortality rates. The third, and unaffected, room contained all the juvenile animals (defined in this study as ≤3yrs) so it is possible that age influenced resistance to overt clinical disease. So although the Jacobson (1980b) and Folsch and Leloup (1976) studies make it tempting to conclude that
ferlaviral transmission within a collection is limited by snake proximity there are confounding variables, such as species and age, that would make this conclusion disputable.

The shedding patterns of ferlavirus are unknown (Jacobson and Origgi, 2007). Although Lloyd and Flanagan (1991) state that some snakes are capable of shedding virus for an extended period of time, this claim is based on the observation that some snakes have significant haemagglutination inhibition (HI) antibody titres for five months or more while simultaneously “causing” seroconversion in cagemates throughout that time. However, it would be difficult to rule out an alternative explanation that all the snakes were infected and took variable amounts of time to seroconvert, especially considering that no screening tests for ferlavirus itself (e.g. virus isolation) were mentioned. Ritchie (2006) suggests that asymptomatic seropositive snakes may be persistently-infected shedders while others may have mounted an appropriate immune response and cleared the infection.

There are currently no reports concerning vertical transmission of ferlavirus (Pasmans et al., 2007).

2.2.6 Treatment and Prognosis

No specific treatment has been identified as being effective against ferlavirus (Marschang and Chitty, 2004). In people, the antiviral drug ribavirin (Virazole®) is sometimes used in the treatment of Measles virus, Respiratory syncytial cell virus and Parainfluenza virus infections (Chakrabarti et al., 2001; Freeman et al., 2004). With the aid of computers, the three-dimensional structure of the neuraminidase protein of an influenza virus was predicted, and based on this, oseltamivir (Tamiflu®) was synthesised as a high-affinity inhibitor of this enzyme (Alymova et al., 2005). Similar technology has been utilised for a drug named BCX 2798 that is capable of targeting paramyxoviral neuraminidase and it has been shown to decrease viral titres in mice infected with a recombinant strain of Sendai virus whose fusion and haemagglutinin-neuraminidase molecules had been replaced with those from Human
parainfluenza virus 3 (Alymova et al., 2005; Watanabe et al., 2009). None of these compounds have been tested against ferlavirus either in vitro or in vivo.

Symptomatic treatment has generally been provided by broad spectrum antibiotics (Kolesnikovas et al., 2006). These may be helpful because the isolation of secondary Gram negative bacteria from ferlavirus-positive snakes has been reported by several authors (Folsch and Leloup, 1976; Jacobson et al., 1981; Jacobson et al., 1992; Homer et al., 1995; Oros et al., 2001; Kolesnikovas et al., 2006; Jacobson, 2007). Bronson and Cranfield (2006) have stated that the survival time of snakes infected with ferlavirus is improved by targeting secondary protozoal and bacterial infections.

The onset of clinical signs, once seen, is usually progressive (Ritchie, 2006). The prognosis in affected snakes should be considered grave and the presence of neurological signs indicates a poorer prognosis (Ritchie, 2006). The anecdotal statement has been made that snakes that become severely lethargic, die within one week of the onset of signs (Bronson and Cranfield, 2006).

### 2.2.7 Prevention

The methods that are commonly used to prevent the spread of contagions in other animals are used in the same way to control the spread of ferlavirus between snakes. The development of an inactivated (killed) ferlavirus vaccine was unsuccessful (Jacobson et al., 1991), so preventing infection is reliant on effective quarantine strategies.

**Quarantine**

To prevent the introduction of ferlavirus into a naïve collection it is recommended that new animals are only introduced after a period of quarantine (Ritchie, 2006). Pasmans et al. (2007) recommends that newly-acquired animals be kept in quarantine terraria that are in a separate room to the resident collection and also that newly-acquired snakes are attended to after
resident ones. It has also been recommended that the resident and quarantined animals are not attended to on the same day and that an entirely different person is used to attend to the resident and quarantined animals (Ritchie, 2006). Surrounding the quarantining period, cages and husbandry equipment should be disinfected (Pasmans et al., 2007). Marschang and Chitty (2004) recommend the use of any virucidal disinfectant. The efficacy of virucidal disinfectants against ferlavirus has not been reported but as a general rule, enveloped viruses are labile to a wide range of disinfectants (Heit and Riviere, 2009).

During quarantine, specific antibody testing can be used to help determine if a snake has been exposed to ferlavirus (Bronson and Cranfield, 2006). Paired samples, taken at an eight-week interval, may reveal an increase in titre indicating a recent infection (Pasmans et al., 2007). Antibody testing is discussed in greater detail in section 2.3.6 – Haemagglutination Inhibition.

The quarantine periods that have been recommended to prevent the introduction of ferlavirus into a collection vary between sources but overall, there is a general trend where modern recommendations are for longer periods of quarantine (Table 2.5). All of the recommendations are empirical due to the limited information that is available about ferlaviral transmission and environmental viability. Also, effective biosecurity necessitates that all the pathogens that can affect an animal’s health are considered. Choosing quarantine periods based on a single pathogen (in this case ferlavirus) is foolhardy. Quarantine periods should be determined by the pathogens with the longest incubation periods. In snakes, ferlavirus is just one infection that requires a lengthy quarantine period. Inclusion body disease (IBD) is another poorly understood infectious disease of snakes where quarantine periods have been reported of at least six months (Keeble, 2004; Schumacher, 2006) and six months to a year (Vancraeynest et al., 2006).
<table>
<thead>
<tr>
<th>Quarantine Time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least 30 days</td>
<td>(Jacobson et al., 1980b)</td>
</tr>
<tr>
<td>Minimum of 60 days</td>
<td>(Lloyd and Flanagan, 1991)</td>
</tr>
<tr>
<td>Minimum of 60-90 days</td>
<td>(Bronson and Cranfield, 2006)</td>
</tr>
<tr>
<td>Up to 90 days</td>
<td>(Gillespie, 2006)</td>
</tr>
<tr>
<td>90 days</td>
<td>(Pasmans et al., 2007)</td>
</tr>
<tr>
<td>Minimum of 90 days for animal raised in captivity</td>
<td>(Ritchie, 2006)</td>
</tr>
<tr>
<td>Minimally 90 days in a clinically healthy collection</td>
<td>(Jacobson et al., 1992; Marschang and Chitty, 2004)</td>
</tr>
<tr>
<td>At least two months since the last death in an affected collection.</td>
<td>(Jacobson et al., 1992)</td>
</tr>
<tr>
<td>Four months</td>
<td>(Hernandez-Divers, 2006)</td>
</tr>
<tr>
<td>Six months or 180 days</td>
<td>(Keeble, 2004; Ritchie, 2006; Rossi, 2006)</td>
</tr>
</tbody>
</table>

Table 2.5 The various quarantine periods that are recommended in the ferlaviral literature.

**Vaccination**

There have only been a few attempts to develop vaccines for reptiles. Yang *et al.* (2007) successfully developed an orally-administered vaccine to protect against *Aeromonas sobria* in soft-shelled turtles (*Trionyx sinensis*). And during several outbreaks of *Mycoplasma crocodyli* in Nile crocodiles (*Crocodylus niloticus*), an autogenous vaccine was produced that was superior to antibiotic therapy in alleviating clinical manifestations of disease (Mohan *et al.*, 1997; Mohan *et al.*, 2001). But a vaccine that is both safe and effective against a reptilian *virus* remains an elusive goal for the researchers that have attempted to develop one (Jacobson *et al.*, 1997; Marschang *et al.*, 2001b; Origgi, 2007). In the only attempt to develop a ferlaviral vaccine, a sustained and significant concentration of circulating anti-ferlavirus antibodies could not be elicited (Jacobson *et al.*, 1991).

In this investigation by Jacobson *et al.* (1991), a black mamba (*Dendroaspis polylepsis*) isolate of ferlavirus was inactivated (killed) using 0.2% beta-propiolactone. This treatment effectively halved the haemagglutinating ability of the virus. The isolate was then treated with aluminum
hydroxide, an oil emulsion, or was left unchanged as a non-adjuvanted virus. Twenty-three western diamondback rattlesnakes (*Crotalus atrox*) were then divided into three groups of six and one group of five. Each of one group of six was injected intramuscularly with a single preparation of ferlaviral vaccine. Snakes in the group of five were inoculated with a control vaccine of *Encephalomyocarditis virus* (EMV) that was inactivated with 0.2% beta-propiolactone and adjuvanted by oil emulsion. At the time of this study, there were no effective reptile vaccines, so EMV was chosen as a control vaccine because it is known to produce antibody responses in mammals that can be detected by haemagglutination inhibition (HI). Vaccines were injected at days 0, 28 and 63. Blood was collected to determine antibody titres by HI at days: -56 (eight weeks prior to vaccination), 0, 14, 28, 42, 63, 84, 136, 176, 219, 253 and 296 days after the first injection of vaccine was given. All pre-vaccination HI titres were zero and a positive titre was defined as being ≥20. The HI titres recorded after vaccinations were variable, unpredictable and could only be detected in seven of the vaccinated snakes (**Table 2.6**). Only one of the vaccinated (oil-emulsion adjuvant) snakes produced a positive HI titre for greater than 200 days. No HI titre could be detected at any time point in the remaining 11 snakes that were vaccinated with ferlavirus or any of the five snakes that were vaccinated with EMV. A small number of snakes stopped eating during the trial but otherwise all remained healthy.
Table 2.6 Adapted from Jacobson et al. (1997). Anti-ferlaviral titres (HI, haemagglutination inhibition) of western diamondback rattlesnakes (Crotalus atrox) after exposure to an inactivated ferlavirus vaccine. All snakes (n=16) and time points (day -56 to day 28) where an HI titre was not detected are not presented and taken as zero.

Protective immunity could not be demonstrated in this study because the vaccinated snakes were not challenged with unattenuated virus. The link between antibody titres and protective immunity has been the source of controversy in small animal medicine (Moore and Glickman, 2004) and in reptiles there is a veritable dearth of information on this important link. The protective immunity against viruses is typically cell-mediated but can sometimes be an uneven contribution of cell-mediated and humoral responses (Wellehan and Johnson, 2005). So the interpretation of haemagglutination inhibition titres as an appropriate surrogate marker for protective immunity in this investigation should not be automatically assumed, especially when biologically relevant (protective) antibody titres are unknown. Furthermore, the secondary immunological response in reptiles may be influenced by a number of factors: inappropriate temperature, type of antigen used, antigenic dose, schedule of immunisation, route of immunisation, seasonal conditions and hormonal conditions (Jacobson et al., 1991). The effect that these variables had on the secondary immune response in this study is difficult to determine. So although Jacobson et al. (1991) concludes that future directions of reptile virus vaccines should consider vaccines with greater antigenic masses and antibody assays
such as enzyme-linked immunosorbent assays (ELISAs) that have greater sensitivities, there are many other considerations that should be investigated as well.

Mayr et al. (2000) has suggested that cell culture-adapted ferlaviral isolates could be used in the production of live vaccines but in a brief and limited report of a vaccine trial using a modified-live isolate of ferlavirus, one snake died and another suffered severe illness (Lloyd and Flanagan, 1991).

2.2.8 Ferlavirus in Non-Captive Free-Ranging Snakes

Only a few reports have described ferlavirus in wild snakes. A survey of 10 free-ranging anacondas (Eunectes murinus) from Venezuela involved serological testing against a number of pathogens, including ferlavirus (Calle et al., 2001). Ferlaviral-specific antibody titres by haemagglutination inhibition (HI) were not detected in any snake.

Allender et al. (2006) screened 20 endangered wild-caught eastern massasauga rattlesnakes (Sistrurus catenatus catenatus) from Illinois for the presence of anti-ferlavirus antibodies using two different isolates of ferlavirus in an HI assay. All the snakes were seropositive against both ferlaviral isolates with titres ranging from 20 to 640. In the absence of additional testing that is capable of detecting ferlavirus (as opposed to just anti-ferlaviral antibodies, e.g. virus isolation, polymerase chain reaction, in situ hybridisation or electron microscopy) the significance of these serological findings is uncertain. Despite this, the authors provide three possibilities to explain the HI results: the snakes were exposed to a non-ferlavirus paramyxovirus that cross-reacts with the HI assay that was utilised; the positive results are false due to the presence of unidentified and non-specific inhibitors in the snakes' sera; or, the snakes were exposed to ferlavirus but were not overtly ill because they had successfully mounted an adequate immune response against the virus.
The results of this report are further complicated by a subsequent serological survey of the same species of free-ranging snakes (*Sistrurus catenatus catenatus*) collected from the same area two years later (Allender *et al.*, 2008). This time, plasma was submitted to three diagnostic laboratories for determination of anti-ferlaviral antibodies by HI using four different isolates of ferlavirus. From 26 snakes sampled, the number of snakes with positive HI titres against each isolate ranged from zero to 26. These results show the considerable disagreement that exists between the HI assays used in this study and the authors conclude that the inter-laboratory variation of this assay makes it an unreliable indicator of infection or exposure to ferlavirus in this species. However, as was discussed earlier (2.1.3 – Specific Information about Ferlaviruses) the various isolates used in each HI assay may be serologically unrelated to each other and would then provide non-comparable results. In summary, using HI as a screening tool to determine if a wild reptile has been exposed to ferlavirus may provide equivocal results.

### 2.2.9 Ferlavirus in Non-Snake Reptilian Hosts

**Chelonians**

In 1983, Jackson and Needham discovered anti-Sendai virus haemagglutination inhibition (HI) titres of up to 256 in 34 tortoises from three species: *Testudo graeca*, *T.hermanni* and *Geochelone elegans*. Seven of these 34 tortoises were showing signs of rhinitis at the time of blood sampling but there was little correlation between anti-Sendai virus HI titre and the presence of clinical signs. The authors did not attempt to isolate a paramyxovirus from any of these tortoises. In another study, a collection of Mediterranean tortoises (*Testudo graeca* and *T.hermanni*) was imported from Turkey to Switzerland and upon arrival many were found to have a viral dermatitis (Zangger *et al.*, 1991). Light microscopy revealed intracytoplasmic inclusions in the stratum germinativum and under electron microscopy; paramyxovirus-like particles were identified.
In 1999, a ferlavirus was isolated from a Hermann’s tortoise (*Testudo hermanni*) suffering from pneumonia (Marschang *et al.*, 2009). The identity of this ferlavirus was confirmed by sequence analysis but no further information is provided. In the most recent chelonian study, several different ferlaviruses were detected by PCR from various organs of a leopard tortoise (*Geochelone pardalis babcocki*) suffering from respiratory distress (Papp *et al.*, 2010b). There were large amounts of mucopurulent discharge from its nares and mouth and on necropsy, the lungs were bilaterally consolidated and filled with thick exudate. The viruses could not be isolated from a range of tissue samples into terrapene heart cells (TH-1) or viper heart cells (VH2).

Based on these few reports it seems that paramyxoviruses are associated with disease in chelonians but it is rarely reported.

**Crocodiles**

Using electron microscopy, paramyxoviruses have been found in the faeces of Nile crocodiles (*Crocodylus niloticus*) (Huchzermeyer *et al.*, 1994). These crocodiles had been fed chickens that were sourced from a farm during an outbreak of Newcastle disease virus. This study also provided limited information about a paramyxovirus that was seen in the faeces of a crocodile not fed a diet of chickens.

**Lizards**

Most of the paramyxoviruses that have been described in lizards have not been associated with overt disease. A paramyxovirus was isolated from a false tegu (*Callopistes maculatus*) in 1988 (Ahne and Neubert, 1991). This paramyxovirus was later identified as a ferlavirus by sequence analysis (Ahne *et al.*, 1999b). Similarly, a paramyxovirus was isolated from the mouth of a healthy monitor lizard (*Varanus prasinus*) that was part of a reptile collection that had suffered an outbreak of ferlavirus in its snake population (Gravendyck *et al.*, 1998).
In a serological survey of lizards, Gravendyck et al. (1998) collected 35 healthy free-ranging Honduran Island iguanas (*Ctenosaura bakeri, C similis and Iguana iguana rhinolophoida*) to look for evidence of paramyxoviral and reoviral infections. Using two reptilian paramyxoviruses as antigens, 41% of serum samples had antibodies that could be detected by virus neutralisation and 9% had haemagglutination inhibition (HI) antibody titres of ≥20 (maximum titre was 32). Sera could not be treated for non-specific haemagglutinins due to the small sample volume and if these were present, they could have potentially decreased the final HI titres. The authors could not isolate any viruses from pharyngeal and cloacal swabs from these lizards. In a similar study, Marschang et al. (2002) tested wild-caught Mexican lizards (*Xenosaurus grandis, X. platyceps and Abronia graminea*) for exposure to paramyxovirus and reovirus. Anti-ferlavirus antibodies were detected in four animals representing all three species but significantly, a ferlavirus was isolated from the cloacal swab of *X. platyceps*. The results of this report were unable to clarify the clinical significance of finding ferlavirus and ferlaviral antibodies in these species.

Lloyd et al. (2005) serologically tested the lizard population of a zoological park where the resident snake collection had a history of paramyxovirus-associated disease. In total, 59 lizards (from 12 families) were tested for the presence of ferlavirus-specific antibodies by haemagglutination inhibition (HI). All the lizards, except one, were clinically normal. Seven lizards had HI titres that were considered positive (≥16) for exposure to ferlavirus. These seven positive animals were then retested 105 days later and six of them had either maintained or increased their antibody titres. The authors concluded that the six animals either had active infection, repeated antigen exposure or were in a carrier state. The authors did not comment on the possibility that the HI titres might be falsely positive. In another study, ferlavirus exposure was serologically assessed in 32 geckos (*Gecko monarchus* and *Gehyra mutilata*) that free-roamed the grounds of a zoological park (Kummrow *et al.*, 2004). Blood was pooled from these small geckos and 70% of pooled blood samples tested positive for ferlavirus exposure by
HI. The authors speculate that the geckos may have a role as vectors for this virus but more
detailed investigations would be necessary to elucidate this idea further.

There are only two reports where a paramyxovirus was associated with mortality in a lizard
(Jacobson et al., 2001a; Boyer et al., 2005). Three separate epidemics of ferlavirus were seen
between 1998 and 1999 in caiman lizards (Dracena guianensis) that had been imported into
the USA from Peru (Jacobson et al., 2001a). Many individuals were found dead or were
anorexic upon arrival. Histopathology showed severe heterophilic and histiocytic pneumonia
and ferlavirus was detected in tissue sections by immunohistochemistry. A virus was isolated
using viper heart cells and electron microscopic examination of cell culture supernatant
confirmed the presence of a paramyxovirus. In the second report, paramyxovirus-like particles
were seen by electron microscopy in the respiratory tract of a Thai water dragon (Physignathus
concinus) (Boyer et al., 2005). Histological assessment revealed a proliferative interstitial
pneumonia and eosinophilic cytoplasmic inclusions of the pneumocytes and pancreatic
ductular epithelium. DNA probes could not detect a reptilian paramyxovirus in paraffin-
embedded sections.

Only a small proportion of the reports about ferlavirus in non-snake reptiles show a strong
association between disease and infection with this pathogen, but there is insufficient data to
rule out the possibility that lizards, chelonians and maybe even crocodiles, play important roles
as reservoir hosts for ferlavirus. This could have important implications when attempting to
eradicate ferlavirus or prevent its introduction in collections that house snakes with non-snake
reptiles.

2.2.10 Zoonotic Potential

Ahne and Mayr (2000) investigated the capability of ferlavirus to infect human blood
mononuclear cell culture at the virus-permissive temperature of 28°C. Viral replication could
not be detected in this cell line. Potgieter et al. (1987) successfully cultured a paramyxoviral
isolate from a snake in BHK-21 hamster kidney cells at 37°C but this study found that the highest haemagglutination titre, the greatest likelihood to grow in cell culture and the most significant cytopathic effects were seen when the isolate had been grown at 30°C. Under the conditions described by other authors, it was found that ferlaviruses did not replicate at 37°C (Clark et al., 1979; Ahne et al., 1999a; Ahne and Mayr, 2000). Clark et al. (1979) comprehensively investigated the susceptibility of mice to infection with ferlavirus. Intracerebral inoculation of ferlavirus into twenty 4-week-old and forty 24-hour-old mice failed to cause any sign of disease during observation for up to one year, nor were signs of histological lesions seen in the brains of animals as they were sacrificed at regular intervals throughout this year of observation. Ferlaviruses that were inoculated intranasally or injected directly into the footpad of adult mice did not result in any clinical or histological evidence of disease.

Based on this information it would seem unlikely that ferlavirus would pose a serious zoonotic risk to human health. Although reptilian paramyxoviruses have been propagated in avian (Ahne et al., 1999a), piscine (Potgieter et al., 1987) and mammalian substrates (Mayr et al., 2000), no species of paramyxovirus has been found that is capable of infecting two different vertebrate classes (e.g. reptiles and mammals) in vivo, so even the upper airways of a human (potentially providing a ferlavirus-permissive temperature) would be unlikely to be colonised or infected.

### 2.3 Diagnostic Tests

#### 2.3.1 Virus Isolation

For paramyxoviruses, virus isolation has traditionally been the “gold standard” diagnostic test that others are compared to (Quinn et al., 2002). The isolation of reptilian paramyxoviruses from clinical samples is reliant on intact virus proliferating in a biological substrate; commonly cultured cells or embryonated eggs (Origgi and Pare, 2007). Tissue samples and oral and cloacal swabs are often used to isolate viruses from infected reptiles (Marschang and Chitty,
2004). **Table 2.7** provides a summary of the techniques that have been used to isolate paramyxoviruses from reptiles.
### Virus Isolation Techniques

#### Successful attempts

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum inoculated directly onto Vero cells</td>
<td>(Nogueira et al., 2002)</td>
</tr>
<tr>
<td>Fulfilling Koch’s postulates: Vero cell-adapted ferlavirus was transmitted to naïve snakes and then lung homogenates were recovered at necropsy and used to reisolate ferlavirus onto Vero cells</td>
<td>(Jacobson et al., 1997)</td>
</tr>
<tr>
<td>Lung suspension inoculated onto fathead minnow skin cells (FHM, a piscine cell line), hamster kidney cells (BHK-21), swine testicular cells (ST), Vero cells and primary bovine turbinate cells (BTU)</td>
<td>(Potgieter et al., 1987)</td>
</tr>
<tr>
<td>Lung suspension inoculated into embryonated snake eggs and then subcultured onto nine reptilian and four mammalian cell lines</td>
<td>(Clark et al., 1979)</td>
</tr>
<tr>
<td>Pooled and/or individual snake organs inoculated onto monolayers of VH2 and/or IgH2 cells</td>
<td>(Jacobson et al., 1980b; Jacobson et al., 1981; Ahne et al., 1987a; Homer et al., 1995; Kolesnikovas et al., 2006; Abbas et al., 2011)</td>
</tr>
<tr>
<td>Various lizard organs inoculated onto VH2 and TH1 cells. After eight passages in TH1 cells, was adapted to Vero cells</td>
<td>(Jacobson et al., 2001a)</td>
</tr>
<tr>
<td>Ferlavirus replicating in VH2 cells adapted to Vero cells</td>
<td>(Richter et al., 1996; Mayr et al., 2000)</td>
</tr>
<tr>
<td>Ferlavirus replicating in IgH2 cells adapted to Vero cells</td>
<td>(Richter et al., 1996; Mayr et al., 2000)</td>
</tr>
<tr>
<td>Ferlavirus replicating in IgH2 cells adapted to chicken embryo fibroblasts (LSCC-H32)</td>
<td>(Ahne et al., 1999a)</td>
</tr>
<tr>
<td>Lung suspension inoculated into snake embryo fibroblasts</td>
<td>(Manvell et al., 2000)</td>
</tr>
</tbody>
</table>

#### Unsuccessful attempts*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tortoise organ suspensions inoculated onto TH1 and VH2 cells</td>
<td>(Papp et al., 2010b)</td>
</tr>
<tr>
<td>Pooled and/or individual snake organs inoculated onto monolayer of IgH2 cells</td>
<td>(Jacobson et al., 1980b)</td>
</tr>
<tr>
<td>Lung suspension inoculated into embryonated snake eggs and then subcultured onto three piscine cell lines</td>
<td>(Clark et al., 1979)</td>
</tr>
<tr>
<td>Lung suspension inoculated into the allantois of SPF embryonated chicken eggs and onto chicken embryo fibroblasts and Vero cells</td>
<td>(Manvell et al., 2000)</td>
</tr>
<tr>
<td>Lung suspension inoculated onto VH2 cells and feline kidney cells (CRFK)</td>
<td>(Potgieter et al., 1987)</td>
</tr>
<tr>
<td>Reptilian paramyxovirus replicating in fathead minnow cells subcultured into the allantois of SPF embryonated chicken eggs</td>
<td>(Potgieter et al., 1987)</td>
</tr>
</tbody>
</table>

*Only those attempts where a paramyxovirus was successfully isolated using a different biological substrate are mentioned. This is to exclude the possibility that the unsuccessful attempt was because there was simply no virus in the inoculum. However, the possibility that the virus did grow in these cells but was not detected cannot be ruled out as further testing (e.g. polymerase chain reaction) was not performed.

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Table 2.7 The methods that have been reported in the literature to isolate paramyxoviruses from reptiles. VH2 = viper heart cells. TH1 = terrapene heart cells. IgH2 = iguana heart cells. SPF = specific pathogen free.
**Cell Culture**

Monolayers of cultured cells can be used to isolate viruses (Origgi and Pare, 2007). A sample is processed for virus isolation and is then added to the cell culture media (the supernatant) of susceptible cells. Any viruses that are present in the sample are given the opportunity to propagate in the cultured cells. The cell types that have been used for the isolation of reptilian viruses are often immortal cell lines: viper heart cells (VH2; American Type Culture Collection [ATCC]-CCL 140) which are fibroblasts from the heart of a Russell’s viper (*Vipera russelli*); iguana heart cells (IgH2; ATCC-CCL 108) which are epithelial cells from the heart of a green iguana (*Iguana iguana*); terrapene heart cells (TH-1; ATCC-CCL 50) which are epithelial cells from the heart of a box turtle (*Terrapene carolina*); or Vero cells (ATCC-CCL 81) which are epithelial cells from the kidney of an African green monkey (*Cercopithecus aethiops*) (Origgi and Pare, 2007; ATCC, 2011). Ferlavirus is usually isolated using reptilian cell lines (most commonly viper heart cells) and in some cases has then been adapted to grow in Vero cells (Richter et al., 1996; Ahne et al., 1999a; Mayr et al., 2000; Jacobson et al., 2001a). In one report, a ferlavirus was isolated from the sputum of a South American rattlesnake (*Crotalus durissus terrificus*) directly into Vero cells (Nogueira et al., 2002).

Cultured cells that are infected with a virus may display signs of their infection (Origgi and Pare, 2007). These signs are collectively termed the cytopathic effect (CPE). The cytopathic effect associated with infection from reptilian paramyxoviruses has been described by a number of authors (Table 2.8). Syncytial cell formation and cell lysis are commonly reported (Figure 2.7). The time taken for CPE to emerge varies markedly between references: from only 24 to 36 hours (Ahne et al., 1987a) to requiring serial passage (Jacobson et al., 1980b).
<table>
<thead>
<tr>
<th>Cytopathic Effect</th>
<th>Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viper heart cells (VH2)</td>
</tr>
<tr>
<td>Cytoplasmic inclusion bodies</td>
<td>[Abbas et al., 2011]³ (Jacobson et al., 1981; Blahak, 1995; Homer et al., 1995; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Cell lysis/monolayer destruction</td>
<td>(Abbas et al., 2011)³ (Jacobson et al., 1981; Blahak, 1995; Homer et al., 1995; Kolesnikovas et al., 2006)</td>
</tr>
<tr>
<td>Elongation of cell processes</td>
<td>(Homer et al., 1995)</td>
</tr>
<tr>
<td>Cell vacuolisation</td>
<td></td>
</tr>
<tr>
<td>Cell rounding</td>
<td>(Kolesnikovas et al., 2006)</td>
</tr>
</tbody>
</table>

Table 2.8 The cytopathic effects of paramyxoviruses isolated from snakes and a lizard that have been described in the literature. §This isolate displayed CPE in nine reptilian and four mammalian cell lines after first being passaged through an embryonated snake egg. ³Adapted from VH2 cells. ²Adapted from IgH2 cells. ¹Adapted from TH1 cells. *Undescribed cytopathic changes were also seen when this isolate was propagated in hamster kidney cells (BHK-21), swine testicular cells (ST), Vero cells and primary bovine turbinate cells (BTU). °This isolate was obtained from a caiman lizard (*Draecena guianensis*). *Tissue homogenates were inoculated onto VH2 and IgH2 cells but cell line that the isolate was successful isolated with is not specified.
Figure 2.7 Both images from Mayr et al. (2000). Top: Normal Vero cells stained with haematoxylin and eosin. Bottom: Vero cells, five days after infection with ferlavirus. Massive syncytial cell formation, lysis of the monolayer and cell process elongation can be seen.


*Embryonated Eggs*

The successful isolation and/or propagation of reptilian paramyxoviruses using embryonated eggs has been reported (Clark *et al.*, 1979; Ahne *et al.*, 1999a). Similar attempts by other authors were unsuccessful (Potgieter *et al.*, 1987; Manvell *et al.*, 2000) (Table 2.7). Clark *et al.* (1979) inoculated lung suspensions into a site near the embryo of three 25-day-old embryonated snake eggs (*Cyclagras gigas*). Each embryo died seven days after infection and the snake embryo was then frozen and processed for inoculation into several vertebrate cell lines and the allantoic and/or amniotic cavities of 7- to 8-day-old embryonated chicken eggs. A ferlavirus was isolated that had a haemagglutination titre of 1:32 (using guinea pig erythrocytes) after incubation in a snake egg. This titre increased up to 1:512 after serial passage in chicken eggs.

Ahne *et al.* (1999a) were able to adapt IgH2-grown ferlavirus to the allantoic cavity of 8-day-old embryonated chicken eggs and obtain tissue culture infectious doses (TCID$_{50}$) of $10^{7.8}$-$10^{8.2}$ mL$^{-1}$ after six days of incubation at 28°C. The minimum infective dose was 0.1 TCID$_{50}$. Using embryonated chicken eggs, Manvell *et al.* (2000) attempted to isolate a ferlavirus, and Potgieter *et al.* (1987) attempted to propagate a reptilian paramyxovirus that had been previously isolated using fathead minnow skin (FHM) cells. Both attempts were unsuccessful. Potgieter *et al.* (1987) was unable to detect haemagglutinating activity in the allantoic cavity of the chicken egg and Manvell *et al.* (2000) do not provide a basis for their unsuccessful isolation.
2.3.2 Electron Microscopy

Many authors have utilised transmission electron microscopy (TEM) to identify reptilian paramyxoviruses (Lunger and Clark, 1978; Clark et al., 1979; Jacobson et al., 1980b; Jacobson et al., 1981; Ahne et al., 1987a; Potgieter et al., 1987; Richter et al., 1996; Jacobson et al., 1997; Manvell et al., 2000; Franke et al., 2001; Jacobson et al., 2001a; West et al., 2001). Ferlaviruses are medium-sized, have spiked envelopes and can be spherical to pleomorphic in morphology (Jacobson and Samuelson, 2007) (Figure 2.8). The nucleocapsid of a paramyxovirus forms the core of the virion and has a distinct “herring bone” appearance (Ahne and Mayr, 2000) (Figure 2.8 bottom). Spherical and filamentous forms of ferlavirus have been seen budding from infected cells (Jacobson et al., 1997) (Figure 2.9).

Inclusion bodies have been identified under light microscopy in snake tissue infected with ferlavirus (Jacobson et al., 1981; Potgieter et al., 1987; Homer et al., 1995; Jacobson et al., 1997; West et al., 2001). Ultrastructural assessment has shown these inclusions to be comprised of nucleocapsid strands (Jacobson et al., 1981; Jacobson et al., 1997) (Figure 2.10).
Figure 2.8 Transmission electron microscopic (negative staining) appearance of ferlavirus. **Top:** Spherical appearance of ferlavirus showing spiked envelope (Ahne et al., 1999b). Scale bar represents 100nm. **Bottom:** Disrupted ferlavirus releasing its characteristic “herring-bone” nucleocapsid protein (Ahne and Mayr, 2000). Scale bar represents 100nm.
Figure 2.9 Transmission electron microscopic (positive staining) appearance of ferlavirus-infected cells. Hyperplastic type II epithelial cells from the lung of an Aruba Island rattlesnake (*Crotalus unicolor*) eight days after experimental infection with ferlavirus (Jacobson *et al.*, 1997). Spherical and filamentous virions can be seen budding from the cell membrane (arrowheads). Scale bar of main picture represents 2µm and scale bar of inset represents 200nm.
Figure 2.10 Transmission electron microscopic (positive staining) appearance of ferlavirus-infected cells. Infected Vero cells showing virions budding from the cell membrane into the intercellular spaces (arrows) (Jacobson et al., 1997). IC = an intracytoplasmic inclusion body containing nucleocapsid material. Scale bar represents 7µm.
2.3.3 Haemagglutination and Haemadsorption Assays

Haemagglutination (HA) assays rely on a sample containing the ability to cause the in vitro macroscopic agglutination of erythrocytes (Jacobson and Origgi, 2007). In virology, the presence of haemagglutination suggests the presence of a haemagglutinating virus, such as a paramyxovirus, (Jacobson and Origgi, 2007) but viruses that are capable of in vitro haemagglutination were only discovered decades after these traits had already been described in bacteria, such as staphylococci and campylobacter (Neter et al., 1954).

Large quantities of virus are needed for macroscopic haemagglutination so this method is considered to be relatively insensitive (Quinn et al., 2002). In one study, ferlavirused-infected tissue homogenates did not haemagglutinate chicken erythrocytes but ferlavirus could be isolated onto viper heart cells and be detected by polymerase chain reaction (Kolesnikovas et al., 2006). Only after replication in viper heart cells, could haemagglutinating activity be detected. Studies comparing the lower limits of detection of haemagglutination to other diagnostic tests, such as polymerase chain reaction, do not exist.

Various vertebrate erythrocytes were compared to each other in their ability to haemagglutinate three isolates of ferlavirus (Richter et al., 1996). Chicken and guinea pig erythrocytes reliably produced high titres against these three isolates and outperformed sheep, human type-O and rabbit erythrocytes. Haemagglutination inhibition is offered commercially at several diagnostic laboratories as a serological test for ferlaviral exposure and many, if not all, of these laboratories utilise chicken or guinea pig erythrocytes as markers of ferlavirus-induced haemagglutination (Allender et al., 2008).

In haemadsorption assays, erythrocytes attach themselves to the surface of a cell monolayer that is infected with a haemagglutinating virus (Mahy and Kangro, 1996). Haemadsorption of
cells infected with ferlavirus has not been reported but it has been described in a paramyxovirus known to infect Atlantic salmon (Kvellestad et al., 2003).

2.3.4 Neuraminidase Assay

Neuraminidase (sialidase, receptor destroying enzyme) is a protein found on the surface of many myxoviruses (orthomyxoviruses and paramyxoviruses) that is capable of cleaving the glycosidic linkages of neuraminic acid (sialic acid) (Clark et al., 1979; Klenk and Herrler, 1997). Paramyxoviral neuraminidase serves two purposes. Firstly, during infection, the attachment protein of a paramyxovirus binds to a sialic acid residue on the surface of the host cell. But this bond can prevent the release of viral progeny from an infected cell so viral neuraminidase is used to cleave the sialic acid bond allowing the viral progeny to be released. This has the added benefit that progeny virions will not reattach themselves to previously infected cells allowing an infection to progress onto naive cells. Secondly, viral neuraminidase will cleave any sialic acid residues from viral proteins. This helps prevent viral aggregation.

Using a substrate that is specifically cleaved by neuraminidase into an intensely fluorescent product (Yolken et al., 1980), significant neuraminidase activity was detected in three isolates of ferlavirus (Richter et al., 1996). Clark et al. (1979) also demonstrated the presence of neuraminidase activity in a ferlavirus. After haemagglutinating chicken and guinea pig erythrocytes with ferlavirus, it was observed that these erythrocytes could not be re-agglutinated by ferlavirus, implying that the virus had a receptor destroying enzyme (neuraminidase).

2.3.5 Non-haemagglutination Inhibition Antibody Assays

The detection of reptilian antibody responses to viruses has been reviewed by Jacobson and Origgi (2007). Serum (virus) neutralisation tests (SN, SNT, VN, VNT) are considered to be the “gold standard” antibody tests. These tests quantify the capability of serum or plasma to neutralise a particular pathogen’s infectivity of a biological substrate, such as a cell line. Serum
neutralisation tests are labour-intensive and often have protracted turnaround times before results are available. In spite of this, SNT has been used for the detection of exposure to ferlavirus (Marschang et al., 2002). Enzyme-linked immunosorbent assay (ELISA) is a relatively modern technique that can also be used for the detection of antibodies (Jacobson and Origgi, 2007). It is highly sensitive but requires reagents that are timely to create (months) and equipment that is expensive (up to US$10,000). Like SNT, ELISA has been used for the detection of ferlavirus exposure (Kania et al., 2000).

2.3.6 Haemagglutination Inhibition

In contrast to serum neutralisation tests (SNT) and enzyme-linked immunosorbent assays (ELISA), haemagglutination inhibition (HI) is a relatively simple, rapid and inexpensive test of humoral immunity (Quinn et al., 2002; Jacobson and Origgi, 2007). In this test, the ability of serum or plasma to inhibit viral haemagglutination is quantified (Jacobson and Origgi, 2007). Haemagglutination inhibition has been used widely as a serological test for the detection of exposure to ferlavirus (Jacobson et al., 1981; Potgieter et al., 1987; Jacobson et al., 1991; Jacobson et al., 1992; Brousset et al., 1994; Blahak, 1995; Richter et al., 1996; Jacobson et al., 1997; Gravendyck et al., 1998; Manvell et al., 2000; Calle et al., 2001; Jacobson et al., 2001a; Marschang et al., 2002; Lloyd et al., 2005; Allender et al., 2006; Allender et al., 2008) and is offered commercially by several diagnostic laboratories (Table 2.9).

Haemagglutination inhibition (HI) titres are represented as the reciprocal of the greatest dilution of serum or plasma that inhibits macroscopic haemagglutination, so if the dilutions of 1:2, 1:4, 1:8 and 1:16, but not 1:32, inhibited macroscopic haemagglutination, the HI titre would be 16 (Jacobson and Origgi, 2007). There is disagreement in the literature about the titres that should be considered positive for exposure to ferlavirus. Titres of greater than 10 (Jacobson et al., 1992) and 16 (Pasmans et al., 2007) have been reported but it has also been suggested that less than 20 is negative, between 40 and 80 is suspect and greater than 80 is positive (Jacobson and Origgi, 2007).
Pasmans et al. (2007) makes the logical but unsupported claim that the presence of circulating anti-ferlavirus antibodies in a snake indicates the survival from infection but the animal may still be carrying latent virus. Pasmans et al. (2007) goes on to recommend that paired samples, eight weeks apart, be taken to determine if a snake has a rising antibody titre. A rising titre may indicate current exposure to ferlavirus, while a “positive” titre that does not increase may be indicative of previous exposure (Jacobson and Origgi, 2007). HI assays quantify the ability of serum or plasma to inhibit macroscopic haemagglutination without delineating the contributions to this inhibition that were made by immunoglobulin M (IgM) and IgY (the reptilian equivalent of IgG). Furthermore, controlled experiments that were able to identify the antibody titres at several time points during and after antigen exposure do not exist. So conclusions drawn from rising, falling or unchanged anti-ferlavirus HI titres may not always be reliable. Although it might be difficult to interpret HI titres, reports do exist that provide useful information about the HI titres that were seen during both a controlled transmission study and naturally occurring outbreaks of ferlavirus infection.

The experimental transmission of ferlavirus in Aruba Island rattlesnakes (Crotalus unicolor) forms the only study where ferlavirus was inoculated into snakes under controlled conditions (Jacobson et al., 1997). In this study, HI titres were only assessed at the time of death. The last death occurred 22 days after inoculation. During this time, no snake had developed an antibody response that could be detected by HI.

In an outbreak of ferlavirus in a zoological collection, Jacobson et al. (1992) tested 31 snakes for the presence of anti-ferlavirus antibodies by HI. Twelve snakes showed positive titres (greater than 10) and these animals were then retested a number of times over the next year. Many cases showed high titres (5,120 to greater than 20,480) that decreased to low titres (below 100) over three to seven months. For example, the titre of a canebrake rattlesnake
(Crotalus horridus) dropped from greater than 20,480 to 20 in a little over seven months and a
gopher snake (Pituophis melanoleucus) had a titre that fell from greater than 20,480 to zero\(^2\) in
approximately one year.

In another outbreak of ferlavirus in reptiles, this time a collection of caiman lizards (Draecena
guianensis), HI testing was performed on surviving animals several months after a ferlavirus
had been isolated from dead animals (Jacobson et al., 2001a). From 17 animals tested, there
were seven titres of less than or equal to 20 and ten titres were between 20 and 180.

An HI titre is complicated by a long list of variables: the antibody’s affinity to the antigen, the
integrity of the antigen being used, the availability of antibody in the serum, the preservation
of the sample and lastly, the host’s immune response, which itself is influenced by
temperature, the season, nutritional status, antigen concentration, route of inoculation,
frequency of exposure to the antigen and the type of antigen (Lloyd et al., 2005). The
influences that these factors have on an HI titre have not been investigated in a controlled
experiment and so the consideration that should be made to each of these factors can only be
speculated.

The time it takes for an animal to mount a detectable immune response to ferlavirus is another
important consideration when testing animals by HI (Lloyd et al., 2005). From the results of a
ferlavirus challenge study that were presented at a conference, it was concluded that naïve
snakes typically take six to eight weeks to seroconvert (Gaskin et al., 1989). It has been
reported that some snakes will die prior to the development of a detectable (by HI) immune
response (Ritchie, 2006). This suggests that testing live or dead snakes by HI shortly after
exposure to ferlavirus may provide negative results. In the period before seroconversion, false

\(^2\) Considering the lower limit of detection of this assay is not known and because serum samples were
initially diluted by a factor of ten, it is unlikely that a titre of zero is accurate.
negatives are a legitimate concern and analysing paired serum samples may help to overcome this hurdle (Ritchie, 2006).

According to Lloyd et al. (2005), if there is a serological unrelatedness between the ferlavirus that has been used as antigen in an HI assay and the ferlavirus the animal has been exposed to, negative HI results may occur. For this reason, other authors have recommended that two different viral isolates are used as the antigen source to accommodate serological differences that might exist between ferlavirus strains (Pasmans et al., 2007). In a study of 60 snake serum samples that were being tested by HI using two different strains of ferlavirus as antigens, considerable variation in HI titre was seen between the two antigens but most snakes that were considered to be positive, were positive using either antigen (Kania et al., 2000).

In contrast to the findings of Kania et al. (2000), Allender et al. (2008) found there was considerable variation in the HI titres of 26 wild-caught eastern massasugas (Sistrurus catenatus catenatus) when analysed using four different strains of ferlavirus as antigen. Against two antigens, 100 percent of plasma samples were positive, 56 percent were positive against the third and none were positive against the last. If all of the HI results that were presented in both the Kania et al. (2000) and Allender et al. (2008) studies were true, then the results may be explained simply by an unrelatedness of the antigens that were being used in the assays. But the possibility that some of these HI results were false cannot be excluded. Irrespective of whether either of these explanations is true, these studies provide further evidence of the difficulties in interpreting HI titres.

Only one investigation has compared HI to another test of humoral immunity: ELISA (Kania et al., 2000). Although there were titre differences between these two diagnostic tests, overall, there was agreement as to whether a sample was positive or negative.
While it seems as though rising or negative HI titres may provide some helpful information to a clinician about exposure to ferlavirus, the variables that confound these results have not yet been adequately studied and so the interpretation of single titres are problematic at best. Some zoological collections and private institutions require negative ferlavirus titres during quarantine before a snake is released into the main collection (Allender et al., 2008) and the difficulties in interpreting HI titres places the decision-making veterinarian in a difficult position.

2.3.7 Immunohistochemistry

In this method, labelled anti-virus antibodies are used to detect the presence of viral antigen in various samples including formalin-fixed paraffin-embedded (FFPE) tissues (Quinn et al., 2002). Immunohistochemistry (IHC) can complement other diagnostic tests because a pathologist can compare the location of immunohistochemical staining to histological lesions. IHC requires the availability of animal-derived polyclonal or monoclonal antibodies that are specific to the virus under investigation (Sand et al., 2004). The use of polyclonal antibodies makes this test difficult to standardise between laboratories (Homer et al., 1995).

The detection of ferlavirus by IHC has been reported by various authors. Homer et al. (1995) inoculated rabbits with ferlavirus to produce a source of anti-ferlavirus polyclonal antibodies. These antibodies were then used to immunohistochemically identify ferlavirus antigen in FFPE tissues. This study demonstrated that standard formalin-fixation practices did not prevent the identification of ferlavirus in infected tissues, although fixation times are not always listed. Since this first report, IHC has been used to detect ferlavirus antigen in ferlavirus-infected Vero cells (Richter et al., 1996), five experimentally-inoculated Aruba Island rattlesnakes (Crotalus unicolor) (Jacobson et al., 1997), a caiman lizard (Draecena guianensis) (Jacobson et al., 2001a), six snakes from the Canary Islands (Oros et al., 2001) and three pit vipers (Bothrops alternatus) from Brazil (Kolesnikovas et al., 2006).
2.3.8 *In Situ* Hybridisation

This method uses a labelled sequence of nucleic acids, called a probe, to anneal, or hybridise, to a target sequence of nucleic acids on a diagnostic sample (Johnson *et al.*, 2007). An advantage that *in situ* hybridisation (ISH) has over IHC, is that biologically-derived polyclonal or monoclonal antibodies are not needed and that viral transcripts can be detected (Sand *et al.*, 2004). Like IHC, ISH can be used to temporally and spatially associate viruses with histological lesions.

Using oligonucleotides as probes, Sand *et al.* (2004) were able to identify segments of the ferlavirus haemagglutinin-neuraminidase attachment gene (HN) in the tissue sections of 11 out of 14 snakes that had histopathological findings that were consistent with a ferlavirus infection. The 14 samples were then tested by reverse transcription polymerase chain reaction (RT-PCR) and the same 11 samples were positive. No further investigations of the three negative results were reported. The detection of a transcript of a ubiquitous house-keeping gene (e.g. 18S rRNA, beta-actin) may have provided an internal control for all the samples. If a sample was negative for both ferlavirus and a house-keeping gene transcript, the integrity of the sample would become doubtful and the negative result for ferlavirus, by both ISH and RT-PCR, would become equivocal.

Only one other report describes the use of ISH for the detection of ferlavirus (West *et al.*, 2001). A generic avian paramyxovirus probe was used for the detection of ferlavirus in the brain of a Boelen’s python (*Morelia boeleni*) with neurological signs. Hybridisation was observed even after increasing the stringency of the procedure.

2.3.9 Polymerase Chain Reaction

Polymerase chain reaction (PCR) involves the geometric amplification of target sequences of DNA using short sequences of synthetic DNA, called primers, such that femtogram ($10^{-15}$g) amounts of target DNA may be detected in microgram ($10^{-6}$g) amounts of total DNA (Johnson...
et al., 2007). RNA viruses, such as ferlavirus, can only be detected by PCR after the viral RNA has been reverse-transcribed into complementary DNA, known as cDNA. The detection of target RNA by PCR is known as reverse transcription-PCR (RT-PCR).

As mentioned in the previous section on in situ hybridisation (ISH), Sand et al. (2004) used RT-PCR to identify ferlaviral RNA in FFPE tissues. Primer sequences that target the attachment gene (HN) were designed that produce relatively small amplicons: 153 nucleotides. Targeting short amplicons increases the likelihood of detecting RNA that may have been degraded during formalin-fixation (Antonov et al., 2005). Sand et al. (2004) amplified and then sequenced appropriately-sized amplicons from 11 out of 14 cases that had histological findings consistent with ferlavirus infection.

Although ISH offers an advantage over RT-PCR because histological lesions can be compared spatially to the presence of viral RNA, RT-PCR offers several advantages over other diagnostic tests, not just ISH. Unlike ISH, RT-PCR can be used on a diverse range of starting materials including blood, body cavity fluids, bronchial secretions/washings and other secretions. Sand et al. (2004) reports that RT-PCR also has some important advantages compared to haemagglutination-inhibition (HI): RT-PCR requires less time, reveals early stage of disease (prior to seroconversion) and does not require paired samples. However, RT-PCR has important limitations that are not described by Sand et al. (2004). For example, RT-PCR is dependent on an adequate quantity and quality of viral RNA being present in the sample. In a hypothetical example where a cloacal swab is being tested by RT-PCR and the swab was collected from a snake infected with ferlavirus that is not shedding ferlavirus into its cloaca, the RT-PCR result will be negative. It is conceivable that evidence of this infection could still be obtained by HI. The shedding pattern of ferlavirus is not known (Jacobson and Origgi, 2007), so RT-PCR results that are negative may not be accurate representations of the animal’s disease status.
In 1999, primer sequences were designed and then successfully used for the detection of the polymerase (L) and attachment (HN) genes of ferlavirus (Ahne et al., 1999b). The L gene primer set has been used for nucleic acid detection by several authors (Ahne et al., 1999b; Nogueira et al., 2002; Marschang et al., 2009) and diagnostic laboratories (Table 2.9). In contrast to this, the haemagglutinin-neuraminidase (HN) gene has been targeted by other investigators (Sand et al., 2004; Kolesnikovas et al., 2006). Kurath et al. (2004) reported the order of conservation between paramyxoviral proteins (most conserved to least conserved): V-carboxy domain > L > M/F > N/HN > V > P. In agreement with this, Kindermann et al. (2001) found the L gene, from a selection of ferlaviruses, to be more conserved than the HN gene.

In one study, a PCR targeting the HN gene was performed on 47 clinical samples (swabs, organs) that had previously been determined to be positive for ferlavirus by an L gene PCR (Papp et al., 2010a). Only 34% were positive when tested with the HN gene PCR. Because this study first screened samples with the L gene PCR, there was no opportunity that a sample could be discovered that was positive by the HN gene PCR and negative by the L gene PCR. In another study, Kolesnikovas et al. (2006) used an HN gene primer set (Ahne et al., 1999b) to successfully amplify ferlavirus from cell culture supernatant and infected tissues. Some tissue samples were negative using this primer set but these results were not pursued. The annealing temperature in this study was set at 50°C but in the original study by Ahne et al. (1999b) the authors stated that the HN gene primer set was not always reliable and often the annealing temperature needed to be lowered to accommodate more difficult targets. Considering that every ferlaviral isolate tested so far by PCR, has been positive by an L gene PCR but some have escaped detection by an HN gene PCR (Ahne et al., 1999b; Franke et al., 2001; Marschang et al., 2009; Papp et al., 2010a), it seems reasonable to target the L gene in preference to the HN gene.
Not all authors have used the primer sets designed by Ahne et al. (1999b). In a retrospective study of 22 snakes from the Netherlands that died with histological findings consistent with ferlavirus infection, ten were positive for ferlavirus using newly designed primers (Kik et al., 2004). This conference abstract did not provide details about the amplicon size, primer sequences or the region targeted by the primer set. Other studies have used novel degenerate primers to target the fusion (F) (Franke et al., 2006) and “unknown” (U) (Marschang et al., 2009) genes of ferlavirus but neither primer set has been used diagnostically.

2.3.10 Commercially Available Diagnostic Tests

The diagnostic tests for ferlavirus that are available to the clinical practitioner are restricted to haemagglutination inhibition (HI), reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation (Table 2.9). These tests are only offered on a commercial basis in Europe and the United States of America. For practitioners from Australia to gain access to these tests, various export permits are needed. For example, Australia has listed all 16 python species that are endemic to this country with the Convention on International Trade in Endangered Species (CITES, 2011). To send a sample from any one of these species from Australia requires a species-specific permit to be obtained from CITES. Moreover, some of these international diagnostic laboratories will need to have the relevant import permits in place before they can receive samples from outside their country. Negotiating these bureaucracies can be time and/or cost-prohibitive.
<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Haemagglutination Inhibition (HI)</strong></td>
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</tr>
<tr>
<td>Hohenheim University, Germany</td>
<td>(Heard et al., 2004)</td>
</tr>
<tr>
<td>Chemical and Veterinary Investigation Office (CVUA) of East Westphalia-Lippe (CVUA), Germany</td>
<td>(R. Marschang, pers. comm.)</td>
</tr>
<tr>
<td>Veterinary Laboratories Agency (VLA), UK</td>
<td>(Keeble, 2004)</td>
</tr>
<tr>
<td>The University of Florida, USA</td>
<td>(Heard et al., 2004)</td>
</tr>
<tr>
<td>The University of Tennessee, USA</td>
<td>(Heard et al., 2004)</td>
</tr>
<tr>
<td>Texas State Diagnostic Laboratory, USA</td>
<td>(Ritchie, 2006)</td>
</tr>
<tr>
<td><strong>Reverse Transcription – Polymerase Chain Reaction (RT-PCR)</strong></td>
<td></td>
</tr>
<tr>
<td>Hohenheim University, Germany*</td>
<td>(R. Marschang, pers. comm.)</td>
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<td>Chemical and Veterinary Investigation Office (CVUA) of East Westphalia-Lippe, Germany*</td>
<td>(R. Marschang, pers. comm.)</td>
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<tr>
<td>The University of Florida, USA*</td>
<td>(Heard et al., 2004)</td>
</tr>
<tr>
<td><strong>Virus Isolation</strong></td>
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<tr>
<td>Hohenheim University, Germany</td>
<td>(R. Marschang, pers. comm.)</td>
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<tr>
<td>Chemical and Veterinary Investigation Office (CVUA) of East Westphalia-Lippe, Germany</td>
<td>(R. Marschang, pers. comm.)</td>
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</table>

*Use the primer pairs designed by Ahne et al. (1999b) targeting the L-gene.

Table 2.9 Diagnostic tests for ferlavirus that are commercially available.

In this chapter, the literature that concerns itself with ferlavirus has been reviewed. As will be shown in the following chapters, this information was drawn upon to make decisions about which diagnostic tests to pursue while attempting to discover a paramyxovirus in an Australian snake, the limitations of these tests and how to interpret test results. In the next chapter, a critical review of the information that has justified this investigation into the paramyxoviruses of Australian snakes is provided.
Chapter 3 – Paramyxoviruses in Australian Snakes

In the previous chapter, the peer-reviewed literature of the paramyxoviruses of snakes was reviewed. Compared to Europe and the USA, there is a relative dearth of information about the paramyxoviruses of Australian snakes. Even though currently-practising Australian reptile veterinarians have been seeing snakes display neurorespiratory signs of disease for the last two to three decades (Johnson, 2011), there have only been a few investigations (informal and formal) into the possible causes of disease. Historically, the tests available to the Australian practitioner to diagnose a viral infection in an Australian snake have either been nonexistent or only available through overseas laboratories. To send a sample to an overseas laboratory can be cost prohibitive, the turnaround times may be unacceptable, and the appropriate permits (e.g. CITES) may need to be in place before the samples can be submitted. As mentioned in the previous chapter, negotiating these bureaucracies can be cost and/or time-prohibitive. Partly because of these diagnostic hurdles, Australian reptilian (not just snakes) virology has remained in its infancy.

This chapter begins with a short review of the peer- and non peer-reviewed reports that describe the viruses that have been found in Australian snakes. This is followed by the unpublished results of overseas diagnostic testing that was performed on Australian snake samples. These are presented with the permission of the submitting veterinarians. Included with these results are summaries of a selection of Australian histopathological reports that show justification for the pursuit of overseas diagnostic testing. The recurring anecdotal reports of snakes displaying neurorespiratory signs that do not respond to supportive care, that sometimes occur in outbreaks, and the results of overseas diagnostic testing, have collectively provided an impetus for a formal investigation of Australian snakes for the presence of paramyxoviruses.
3.1 Australian Snake Virology (excluding paramyxoviruses)

In Australia, there are varying degrees of evidence to support the inclusion of iridoviruses, adenoviruses, inclusion body disease (IBD), reoviruses and herpesviruses as endemic viral diseases of native Australian snakes. The evidence for the presence of paramyxoviruses in Australian snakes will be reviewed in the next sections.

In 1998, ten green tree pythons (*Morelia viridis*) were seized at an Australian international airport having been illegally imported from Indonesia. A ranavirus (family: *Iridoviridae*) was isolated from two of these snakes and was associated with rhinitis, pharyngitis and liver necrosis (Hyatt *et al.*, 2002). Although green tree pythons are endemic to Australia and the virus was isolated and identified in Australia, the snakes were of Indonesian origin and had been isolated in quarantine since being seized. However, this paper also provides the unsupported claim that an erythrocytic iridovirus infection has been detected (details not provided) in an Australian diamond python (*Morelia spilota spilota*).

In 2011, an adenovirus (GenBank accession number: JN418933) was detected by the author using polymerase chain reaction (PCR) on pooled organs (kidney, liver and lung) from a captive Australian death adder (*Acanthophis antarcticus*) with lethargy, flaccid paralysis and proliferative interstitial pneumonia (unpublished data).

In 1998, Carlisle-Nowak *et al.* reported on inclusion body disease (IBD) in a diamond python (*Morelia spilota spilota*) and a north-west carpet python (*Morelia spilota variegata*). Diagnosis was based on non-specific and neurological clinical signs and eosinophilic intracytoplasmic inclusion bodies that were seen histologically: both findings were consistent with IBD. The aetiological agent(s) that is responsible for IBD has not yet been definitively determined and so diagnosis of this disease is defined by histopathology (Chang and Jacobson, 2010). In the investigation by Carlisle-Nowak *et al.*, there was no specific testing to attempt to rule out
known viral diseases that could resemble the changes seen in these two snakes (e.g. reovirus or paramyxovirus infection). The two cases presented in this paper have been cited as evidence of IBD in Australia (Oros et al., 1998; Jacobson et al., 2001b; Raymond et al., 2001; Huder et al., 2002; Chang and Jacobson, 2010) but until more specific testing becomes available, the presence of IBD in Australia should not be seen as irrefutable. An immunoassay capable of detecting a 67kD protein that is unique to cases of IBD (Chang and Jacobson, 2010; Chang et al., 2011), may allow a more definitive diagnosis of IBD to be made in the future.

In a separate report about IBD, a group of 40 clinically-normal snakes being held at Taronga Zoo, New South Wales (NSW), on behalf of the NSW National Parks and Wildlife Service, were euthanased after two of these snakes were diagnosed with IBD (Rose and Humphreys, 2002). Electron microscopy had revealed C-type retroviral particles in the tissues from these two snakes. Ten of the 40 snakes were subject to post mortem examination and all had histological evidence of intra-cytoplasmic inclusion bodies within the central nervous system.

Finally, electron microscopy was able to identify reovirus and herpesvirus particles in the brains of Australian snakes with neurological dysfunction (Rose et al., 2005). Further details are not provided.

Prior to the findings presented in this thesis, there have been two levels of support for the presence of paramyxoviruses in Australian snakes. The first level of support consists of clinical signs and pathological findings that are consistent with paramyxovirus infection. These findings do not rule out the infection. The next level of support consists of the results from haemagglutination inhibition assays and in situ hybridisation that are suggestive of paramyxovirus infection. These findings are distinguished from those that are consistent because they are agent-specific.
3.2 Findings Consistent with Paramyxovirus Infection in Australian Snakes

There have been several anecdotal reports from Australian veterinarians, working in both private practice and with public collections, of snakes with neurological dysfunction such as the inability to right itself, opisthotonus, head tremors and ataxia (Figure 3.1). Respiratory clinical signs are reported less frequently. Mouth gaping is sometimes described (Figure 3.2) but it is unclear whether this suggests a neurological and/or respiratory sign of disease. If these cases were associated with paramyxovirus infections, the predominantly neurological signs being seen in Australia would be in contrast to those reported in overseas descriptions of ferlavirus infection: which most commonly are localised to the respiratory system and only occasionally the nervous system (Jacobson, 2007). The majority of these snakes seen in Australia also have non-specific signs of disease such as regurgitation, weakness, lethargy and inappetence. Many of them do not respond to supportive care (e.g. fluid therapy, antibiotics, antiprotozoals and anthelmintics) and not uncommonly, in-contact animals are similarly-affected. A long list of differential diagnoses should be considered for a snake that has non-specific signs of disease, neurological and/or respiratory signs of disease (Girling and Raiti, 2004). So although the diagnosis of a paramyxovirus infection cannot be made on clinical signs alone, they are consistent with this infection. Together, the lack of response to the therapy that was targeted at other infectious aetiologies and the apparent transmissibility of illness, increase the likelihood of a viral infection.
**Figure 3.1** Australian pythons with neurological signs of disease. **Top:** Wild coastal carpet python (*Morelia spilota mcdowelli*) with neurological signs presented to Wildlife Warriors in Queensland. Photo courtesy of Dr Jon Hanger. **Middle:** Captive diamond python (*Morelia spilota spilota*) presented to a private practice in Victoria. Photo courtesy of Dr Tristan Rich. **Bottom:** Captive south-west carpet python (*Morelia spilota imbricata*) presented to the author in Western Australia.
Figure 3.2 A captive spotted python (Antaresia maculosa) presented to a private practice in Queensland. An abnormal posture and inability to right itself (above) was seen along with open mouth breathing (below). It is unclear whether the open mouth breathing was representative of respiratory and/or neurological disease (e.g., abnormal mouth gaping). Photo courtesy of Dr Bob Doneley.
Some of these snakes have been submitted to pathology laboratories for further investigation.

Only some of the pathology reports include gross pathological descriptions (Figures 3.3 and 3.4). Presumably, this is because only tissue samples (as opposed to whole animals) are submitted to pathology laboratories in the majority of cases. The limited information that is available about the gross pathology that is associated with these clinical presentations is non-specific in all of the limited number of reports available to this review. This means that it is likely that gross pathology will not rule out a paramyxoviral infection.

There are numerous histopathological reports of Australian snakes that support the tentative diagnosis of a paramyxoviral infection (Figure 3.5). As was mentioned in Table 2.2, inclusion body disease (IBD) and reovirus infection would be important differential diagnoses for snakes displaying clinical signs of neurorespiratory disease. The neurohistopathology that has been reported for IBD (Schumacher et al., 1994; Carlisle-Nowak et al., 1998; Jacobson et al., 2001b; Vancraeynest et al., 2006) overlaps with the reports described in Pathology Reports 1-4 (Figures 3.3-3.5). However, in cases of IBD, bright intracytoplasmic eosinophilic inclusions are often seen in the epithelial cells of a variety of tissues (Schumacher et al., 1994; Carlisle-Nowak et al., 1998; Chang and Jacobson, 2010), and these changes are not reported in Pathology Reports 1-4. Reovirus infection has been associated with neurological dysfunction in snakes (Vieler et al., 1994) but in this report the brain was not examined histologically as it was used exclusively for virus isolation. The author is not aware of any published reports about reovirus neurohistopathology in snakes.

There are non-infectious explanations for the clinical signs, gross pathology and histological findings that have been reported for these Australian cases. While organophosphate toxicity and deficiencies in thiamine (Vitamin B₁) and vitamin E may provide similar neurological clinical signs and neurohistopathology (Maxie, 2007), it would be difficult to accept that dietary deficiencies or toxin exposure could explain the apparent transmissibility or the geographical...
distribution of disease that has been seen. Also, these particular non-infectious differential diagnoses do not explain the respiratory lesions seen, so other disease processes would need to be considered concurrently.

In summary, the clinical signs, gross pathology and histopathological findings described in this chapter provide evidence that is consistent with the conclusion that paramyxovirus infection is present in Australian snakes. Paramyxoviral infections do not produce pathognomonic histological lesions so while the histological findings may provide the most specific, and therefore compelling, evidence of paramyxovirus involvement so far, the assessment is not agent-specific and so the results should not be interpreted as being definitive. Some Australian clinicians have submitted samples to overseas laboratories for agent-specific testing and these are reviewed in the next section as findings that are suggestive of paramyxovirus infection in Australian snakes.
PATHOLOGY REPORT 1

Euthanased Y/N: Yes, intracardiac barbiturate
Hx: 1 kidney removed approx 2 years ago (granuloma), weak ever since.
Body condition: Fair
External examination: Blisters, now ruptured and dried along dorsum
Body cavities: NAD
Gastrointestinal: NAD, hair masses in rectum from recently digested meal
Respiratory: NAD
Musculoskeletal: Urate tophi at each intervertebral space along spine
Urogenital: Urate visible in ureters and collecting ducts. Spherical 1.5cm diameter mass in kidney
Neurological: NAD
Cardiovascular: Damage from cardiac puncture (1. last week for CBC & MBA and 2. euthanasia). This damage could obscure other path?
Reticuloendothelial: ?Unfamiliar
Endocrine: ?Unfamiliar
Tentative Dx: Renal failure, "spinal gout" could explain lethargy.

GROSS PATHOLOGY:
Multiple tissues including skull and anterior spinal column. One of the soft tissues contains a 15 mm diameter spherical well circumscribed tumour composed of pale semi-firm tissue.

HISTOPATHOLOGY:
In the brain, particularly in the brainstem region and spinal cord, there are marked degenerative and mild inflammatory changes. These are characterised by spongiosis, neuronal necrosis, axonal swelling and infiltration by macrophages containing ceroid pigment, these tending to be concentrated in perivascular locations. Many residual nerve cells have intracytoplasmic accumulation of similar pigment. A solitary eosinophilic intranuclear inclusion body is detected within an astrocyte. The kidney has fibroplasia and cystic tubular dilation with intraluminal accumulation of urates. There is moderate diffuse interstitial infiltration by lymphocytes, histiocytes, plasma cells and heterophils. The abdominal tumour is an encapsulated accumulation of degenerate adipocytes. Examination of oesophagus, intestine and trachea is non-contributory.

DIAGNOSES:
1: Demyelinating encephalomyelitis consistent with paramyxoviral (OPMV) infection
2: Chronic nephritis
3: Mesenteric lipoma

COMMENTS:
1: The demyelinating nature of the encephalomyelitis and the detection of a typical inclusion body indicate paramyxoviral (OPMV) infection.
2: In keeping with the previous finding of a renal granuloma, there is an end-stage kidney related to a chronic inflammatory process.
3: The mesenteric lipoma is an incidental finding.

Figure 3.3 Pathology report of a python (species unknown) which has been weak since a nephrectomy had been performed two years earlier. The histological findings are consistent with a paramyxoviral infection but the gross pathological changes are non-specific. NAD = no abnormalities detected. CBC = complete blood count. MBA = mixed bile acids. OPMV = ophidian paramyxovirus (ferlavirus). Sections of particular interest are highlighted in red.
PATHOLOGY REPORT 2

HISTORY:
Neuro signs and breathing difficulty approx. 1 month. Was treated by another vet with baytril and clav – no improvements. Strongly suspect OPMV. Owner has lost other snakes with similar signs recently.

NECROPSY:
One python carcass, approx. 2.2m body length was submitted for necropsy. The python was in excellent body condition, with abundant abdominal fat stores and good muscling. Moderately-sized testes confirmed the sex as male. The gastrointestinal tract was largely empty apart from scant hair and brown mucus in the stomach and scant liquid brown faeces in the distal intestine. The liver was pale brown with a slight zonal pattern (likely incidental physiological lipidosis). The lungs were bright red and wet on section, suggestive of congestion and oedema. There were no other notable gross abnormalities.

Comment: The cause of death is not apparent from gross necropsy, although many diseases in snakes, including bacterial septicemia and viral diseases often leave few gross lesions. A wide range of tissues, including brain and spinal cord, will be examined histologically for microscopic evidence of disease. A range of tissues has been stored frozen in the event further testing (eg. culture) is deemed necessary based on the histological findings.

HISTOPATHOLOGY:
Tissues are well preserved, except the intestine, which is moderately autolysed.

Brain and spinal cord: In the brain, involving mainly the midbrain and medulla oblongata, there is marked irregular vacuolation of the white matter, progressing to cystic cavitation in the most severely affected foci. There is moderate gliosis throughout the affected white matter, as well as the adjacent grey matter. The gliosis is most pronounced in the vicinity of blood vessels. Rare parenchymal blood vessels exhibit mural fibrinoid change, and many vessels are surrounded by clear space and/or eosinophilic protein droplets (oedema). There are occasional phagocytes containing globular golden-brown pigment in the parenchyma, and a cluster of these cells in the vicinity of a small focus of haemorrhage near the meninges. There is moderate meningeal infiltration with lymphocytes and plasma cells. The spinal cord exhibits similar changes of vacuolation of white matter, with phagocytes occasionally present within vacuoles, and gliosis of both white and grey matter.

Lung: The lung is diffusely congested. There is diffuse mild infundibular mononuclear cell infiltration. The columnar epithelium lining the major airway is moderately infiltrated with lymphocytes and there are scattered necrotic cells within the epithelium. The trachea is more mildly similarly affected.

Spleen: The spleen is large and contains a markedly active lymphoid population consisting primarily of solid sheets of lymphoblasts intermingled with lesser amounts of small lymphocytes and tingible body macrophages.

Thymus: Several thymic lobes are appreciable. Lobes lack a corticomедullary distinction and are well populated with lymphocytes.

Kidney: There is moderate generalised global glomerulosclerosis with mild periglomerular and interstitial fibrosis.

Liver: There is moderate diffuse hepatocellular lipidosis and small clusters of macrophages containing pale brown pigment scattered throughout the parenchyma.

Heart: There is mild lymphoplasmacytic infiltration of the epicardium and myocardium. There are no notable findings in the stomach, thyroid (inactive), testis (active), adrenal gland, pancreas or intestine.

Comment: The brain lesion of moderate nonsuppurative meningoencephalitis with marked white matter vacuolation is most suggestive of viral infection. Ophidian paramyxovirus is the most likely agent, with inclusion body disease of boids (a retrovirus) a less likely alternative, based on the lack of typical inclusions. Either of these viruses may cause primarily CNS lesions and both are accepted as likely being in Australia, although confirmatory diagnostics are not currently readily available. Another possible aetiology for this CNS lesion is organophosphate toxicity (the likelihood of this would need to be judged in view of the history of possible exposure). Finally, and least likely, thiamine or vitamin E deficiencies could conceivably cause these CNS lesions (“thiamine” deficiency is usually either associated with fish-eating species or addition of sulphur to the diet, while vitamin E deficiency is associated with feeding spoiled or overly fatty feed and would normally have steatitis as an accompanying lesion). The other findings in this snake of moderate nonsuppurative bronchointerstitial pneumonia, mild nonsuppurative myocarditis, and possibly even the lymphoid hyperplasia, are also somewhat suggestive of a viral aetiology. The renal lesions are likely incidental and age or diet related.

DIAGNOSIS:
Glomerulosclerosis, lymphoid hyperplasia, mild interstitial pneumonia, nonsuppurative meningoencephalitis Suspect ophidian paramyxovirus

Figure 3.4 Pathology report of a python (species unknown) with neurorespiratory signs. In-contact snakes had been similarly affected and this snake did not respond to a course of enrofloxacin (“baytril”) and amoxicillin-clavulanic acid (“clav”). The necropsy identifies congestion and oedema in the lungs. The histopathology is consistent with ferlaviral (ophidian paramyxovirus) infection. Other possibilities are considered to explain the neuropathology. Sections of particular interest have been highlighted in red. OPMV = ophidian paramyxovirus (ferlavirus). CNS = central nervous system.
PATHOLOGY REPORT 3

HISTOPATHOLOGY:

Brainstem and spinal cord have spongiosis, dystrophic axons, apoptotic necrosis of oligodendroglia, loss of glial cells, astrocytosis, microgliosis, macrophages containing ceroid pigment and eosinophilic intracytoplasmic inclusion bodies in neurons and astrocytes. Ceroid pigment is focally evident in the neuropil and within oligodendrogial cytoplasm.

The lung has congestion and oedema together with interstitial lymphocytic infiltration. There is abundant bile pigment in renal tubular epithelium. Glomeruli have membranous expansion of mesangial matrix. In the liver, hepatocytes have diffuse fatty change and cholestasis is manifested by accumulation of bile in hepatocyte cytoplasm, in canaliculi and in aggregations of sinusoidal macrophages. The spleen has mild lymphoid depletion. Examination of heart, intestine, and stomach, pancreas and ovary is non-contributory.

DIAGNOSIS:
Demyelinating encephalomyelitis consistent with paramyxoviral (OPMV) infection

COMMENTS:
The demyelinating character of the encephalomyelitis and the inclusion bodies indicate OPMV. The interstitial pneumonia, glomerulonephritis, cholestatic hepatopathy, bilirubin nephrosis and splenic lymphoid depletion are quite typical.

PATHOLOGY REPORT 4

HISTOPATHOLOGY:
The sections examined were liver, lung, brain, kidney, testicle, larynx, pancreas, heart, stomach and abdominal fat.

Brain: Capillaries in the brain are congested and many are distended with serum. The grey matter of the brain, especially the medulla, and the spinal cord shows spongiosis with numerous swollen axons and a patchy gliosis. There is also demyelination and spongiosis of the spinal cord white matter with scattered dye gesturing chambers containing macrophages. Neurones in the medullary area are surrounded by and sometimes contain vacuoles. Eosinophilic intranuclear inclusion bodies are numerous.

Lung: The lung is congested with proliferation of alveolar sites and thickening of septae, which contain scattered heterophils and mononuclear inflammatory cells. Hyaline membranes are present on the surface of some alveoli. One corpora amylacea was seen and there are scattered pigmented bearing macrophages in the interstitium.

Larynx: There is a fibrotic and centrally calcified nodule in the soft tissue adjacent to the larynx. In the surrounding connective tissue there is a diffuse infiltration by mixed inflammatory cells principally heterophils and macrophages. There is also some haemorrhage.

No significant lesions were seen in the other organs examined.

DIAGNOSIS:
Demyelination and neuropathy associated with intranuclear inclusions
Mild interstitial pneumonia -- consistent with ophidian paramyxovirus infection of snakes.

COMMENTS:
The changes in the CNS and lung are typical of paramyxovirus infection in snakes. The chronic inflammatory nodule adjacent to the larynx is likely and incidental and probably associated with an old chronic abscess or similar lesion.

Figure 3.5 Two histopathology reports of pythons (species unknown) with histories of neurorespiratory disease. In both cases, a demyelinating neuropathy and interstitial pneumonia was reported. These changes are consistent with paramyxoviral infection. Sections of particular interest have been highlighted in red.
3.3 Findings Suggestive of Paramyxovirus Infection in Australian Snakes

The findings that are suggestive of a paramyxovirus infection in Australian snakes are being distinguished from those that are consistent with infection on the basis that a suggestive finding involves agent-specific testing. Some Australian veterinarians have pursued agent-specific testing by submitting serum samples to the Texas Veterinary Medicine Diagnostic Laboratory (TVMDL) for the detection of anti-ferlavirus antibodies by haemagglutination inhibition (HI), while in other cases, unstained fixed sections have been submitted to the University of Georgia for the detection of a segment of the ferlaviral genome using in situ hybridisation (ISH). The methods of both tests have been published: the HI methods used by the TVMDL fall under the heading “Laboratory 1” in the report by Allender et al. (2008) and the ISH methods are described by Sand et al. (2004).

Haemagglutination Inhibition

From 2007 to 2008, 65 snakes from four Australian zoological collections (details anonymous), in which outbreaks of neurorespiratory disease were occurring, were tested for antibodies against a western diamondback rattlesnake (Crotalus atrox) strain of ferlavirus by haemagglutination inhibition (HI) (unpublished data). The results are summarised in Table 3.1. Thirteen snakes (20%) had titres that were less than 16 and, according to the diagnostic laboratory, were deemed to be negative for prior exposure to ferlavirus. The remaining 52 snakes (80%) had titres ranging from 16 to 256, indicating prior exposure to ferlavirus. The percentage of animals that were positive was reasonably consistent between collections: collection 1 = 8/10 = 80%; collection 2 = 27/33 = 82%; collection 3 = 11/15 = 73%; collection 4 = 6/7 = 86%.
<table>
<thead>
<tr>
<th>HI Titre</th>
<th>Laboratory Interpretation of Titre</th>
<th>Number of Snakes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Collection 1</td>
<td>Collection 2</td>
</tr>
<tr>
<td>&lt;16</td>
<td>Negative</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>Very weak positive – previous exposure / non-specific</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>32</td>
<td>Weak positive – previous exposure / non-specific</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>64</td>
<td>Positive – recent / current exposure</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>128</td>
<td>Strong positive – recent / current exposure</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>256</td>
<td>Very strong positive – recent / current exposure</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
<td><strong>33</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

**Table 3.1** Results of 65 serum samples from four Australian zoological collections that were sent to Texas Veterinary Medicine Diagnostic Laboratory for the detection of anti-ferlaviral antibodies by haemagglutination inhibition (HI). No titre exceeded 256.

On the surface, these results are significant for two reasons. Firstly, there are 52 (out of 65) serum samples that this laboratory defines as being positive for the presence of significant concentrations (HI titre ≥ 16) of anti-ferlaviral antibodies, and furthermore, four of these 52 samples have titres of 256 (“very strong positives”). If the positive predictive value of this assay is acceptable, then this is strong evidence for the presence of ferlavirus in Australian snakes.

Secondly, if ferlaviruses are endemic to Australia, at least one endemic strain is serologically related to the antigen used in the TVMDL assay. This could facilitate the local detection of anti-ferlaviral antibodies in Australian snakes if this isolate was made available to an Australian diagnostic laboratory.

However, there are at least two reasons to question the significance of these results. Firstly, there are no published reports that these HI titres have been correlated with virus neutralisation (VN) titres or experimental infections: the former is believed to provide a more accurate assessment of humoral immunity (Jacobson and Origgi, 2007) and only the HI assay
offered by the University of Florida has been validated with animals that were experimentally infected (Jacobson et al., 1997). So although the methods of the Texan laboratory have been subjected to peer-review (Allender et al., 2008), the author is unaware of any published reports about the validation of their assay. Secondly, samples from the 52 Australian snakes with significant concentrations of anti-ferlaviral antibodies, were not scrutinised for the presence of ferlavirus using methods such as PCR or virus isolation. Detection of the virus itself would not only definitively answer the question of whether paramyxoviruses are endemic to Australian snakes but would also provide support for the significance of antibody titres performed in the future.

**In situ Hybridisation**

In 2009, formalin-fixed paraffin-embedded samples from 18 Australian snakes from Collection 4 (Table 3.1) that died with clinical signs and histopathological findings consistent with a paramyxoviral infection, were sent to the University of Georgia for detection of ferlavirus using *in situ* hybridisation (ISH) (unpublished data). The lung of a coastal taipan (*Oxyuranus scutellatus scutellatus*) and the gall bladder of a green tree snake (*Dendrelaphis punctulata*) were positive while all other tissues were negative. In the publication that describes the methodology of this assay (Sand et al., 2004), a collection of samples were tested by both ISH and polymerase chain reaction (PCR): from 14 samples, 11 were positive by both ISH and PCR, and three were negative by both ISH and PCR. This provides support that this assay may have a high positive predictive value.
3.4 The Status of Paramyxovirus in Australian Snakes Based on the Results of Others

From the balance of information that has been reviewed in this chapter it would seem likely that paramyxoviruses are present in at least some Australian snakes. Ferlaviruses have been isolated from at least one green tree python (*Morelia viridis*) (Allender *et al*., 2006) and at least one carpet python (*Morelia spilota*) (Ahne *et al*., 1999b). This demonstrates the susceptibility of at least two Australian snake species to ferlaviral infection. Additionally, ferlaviruses have been found in a range of elapids, pythons and colubrids (Ahne *et al*., 1999b). These three families represent the majority of snake species found in Australia (Wilson and Swan, 2003). So there is strong evidence supporting the susceptibility of Australian snake species to paramyxoviruses.

As described earlier, Australian snakes have displayed clinical signs that are consistent with a paramyxovirus infection, and more importantly, pathological examination of the tissues from these affected animals has also supported the diagnosis of this infection. Agent-specific testing for anti-ferlaviral antibodies (by HI) and for the detection of sections of the ferlaviral genome on formalin-fixed paraffin-embedded tissues (by ISH), have both provided results that were suggestive of ferlaviral infection. In contrast to these findings, paramyxoviruses have not been detected in Australian snake samples by virus isolation, electron microscopy or sequenced PCR amplicons.

Positive control material was needed for the investigations presented in this thesis and without the isolation of a paramyxovirus in Australia, isolates from overseas needed to be imported. Prior to the importation of a ferlavirus, the appropriate permits from the Australian Quarantine and Inspection Services (AQIS) needed to be in place. Ferlavirus had not previously been imported into Australia and so AQIS sought advice from Biosecurity Australia (BA), a unit in the Federal Department of Agriculture, Fisheries and Forestry (DAFF). As there were not any
peer-reviewed publications describing the detection of ferlavirus in an endemic Australian snake, ferlavirus was considered to be exotic to Australia.

From this review of the results specific to paramyxovirus infections in Australian snakes, a series of questions remain unanswered. Are paramyxoviruses found in Australian snakes? If so, are these paramyxoviruses from the ferlavirus genus? Will the overseas diagnostic tests provide reliable results for Australian samples? Are other viruses, either on their own, or in combination with paramyxoviruses, contributing to at least some of these clinical cases? These questions formed the basis for the aims of the project that were presented on page 1.

The following chapters outline the formal investigations that were performed on Australian snake samples to try and answer these questions and address the aims of this project.
Chapter 4 – Development and Validation of Methods

In this chapter, the materials and methods are discussed. Some of the generic methods presented were developed further to accommodate the specific needs of this project. Any experiments that were performed on positive control material to validate these methods are presented in this chapter but the analysis of samples is reported in subsequent chapters.

4.1 Sample Collection

Samples were received in the form of swabs, blood or tissue (Figure 4.1). For swabs, oral and cloacal swabs were collected. For each swab, veterinarians were instructed to use a sterile cotton-tipped applicator pre-moistened with viral transport medium (= cell culture medium = minimum essential Eagle’s medium with Earle’s salts [MEM, Sigma, cat. no. M5650] supplemented with 5% (v/v) fetal bovine serum [FBS, GIBCO, cat. no. 10100-147], enrofloxacin [25µg/mL using Baytril® 2.5% Oral Solution, Bayer], amphotericin B [5µg/mL, GIBCO, cat. no. 15290-018], penicillin G/streptomycin [50IU/mL and 0.5mg/mL respectively, Sigma, cat. no. P4333-20ML] and L-glutamine [2mM, GIBCO, cat. no. 25030-149]), Hartmann’s solution or 0.9% (w/v) isotonic saline to swab the oral cavity or the cloaca. For oral swabs, veterinarians were asked to ensure that the glottis was swabbed and if possible, also the endotracheal surface. An ear, nose and throat (ENT) swab was sometimes used to swab the trachea of small snakes. Swabs were then placed into small sterile containers such as a 1.5mL Eppendorf tube. 0.7mL plain blood collection tubes were more readily available to some veterinarians and so were occasionally used. The swab was then submerged in Hartmann’s solution, isotonic 0.9% (w/v) saline or viral transport medium.

Some snakes were sampled with three swabs: a combined cloacal and oral swab, a cloacal-only swab, and an oral-only swab. For these snakes, the cloacal-only swab was a swab of the distal cloaca and the oral-only swab was of one side of the mouth. The third swab (combined oral
and cloacal swab) was performed on the other side of the mouth and a slightly more proximal area (i.e. further away from the vent) of the cloaca.

For blood, whole blood, plasma or serum was submitted. For whole blood, the blood was spotted on filter paper and submitted for elution of antibodies. For serum, whole blood was allowed to clot and then centrifuged to allow retrieval of the cell-free supernatant. For plasma, whole blood was anti-coagulated in lithium heparin and then centrifuged to allow retrieval of the cell-free supernatant.

For tissue, samples from various organs were collected aseptically into labelled sterile tubes e.g. urine sample containers.

Samples were then sent to the author by express couriers using either Australia Post or privately-owned courier companies (e.g. TNT, Logical Freight Solutions). Some samples were sent frozen using dry ice, others were submitted in Eskies with ice bricks and occasionally samples were submitted under ambient conditions.
4.2 Virus Isolation and Propagation

Cell culture was used to propagate viruses from viral stocks and to isolate viruses from primary samples. The allantoic cavities of embryonated chicken eggs were only used to propagate virus stocks.

4.2.1 Cell culture

For virus isolation, a number of methods were trialled but ultimately the methods described by Origgi and Pare (2007) were adapted for use as it was found that they provided the best balance between ease, speed and risk of contamination. Swabs and organ samples were used
for virus isolation. Oral and cloacal swabs were submitted in Hartmann’s solution, isotonic saline or viral transport medium (= cell culture medium). Each swab was vigorously vortexed in its media, the swab was removed and the media was then clarified (2,000g for 10 minutes at 4°C). The supernatant was collected and in some cases was then passed through a 0.45µm syringe-tip filter (Millipore, Massachusetts) to remove contaminating bacterial and fungal elements. This supernatant was then ready to be pipetted onto cultured cells.

Organ samples were collected from animals aseptically. The surface of the skin was cleaned with 70% (v/v) alcohol and the skin was incised using sterile instruments (e.g. forceps, scissors and/or scalpel blade). The instruments were then cleaned with 70% alcohol, dried with a paper towel and then flamed with a Bunsen burner. The coelomic cavity was then penetrated using these re-sterilised instruments. The instruments were sterilised again and used to retrieve the required organ samples. Organ samples (approximately 1cm³) were individually placed into 2mL aliquots of virus isolation media (minimum essential Eagle’s medium with Earle’s salts [MEM, Sigma, cat. no. M5650] supplemented with 5% (v/v) foetal bovine serum [FBS, GIBCO, cat. no. 10100-147], 2x enrofloxacin [25µg/mL using Baytril® 2.5% Oral Solution, Bayer], 2x amphotericin B [5µg/mL, GIBCO, cat. no. 15290-018], 5x penicillin G/streptomycin [50IU/mL and 0.5mg/mL respectively, Sigma, cat. no. P4333-20ML] and 1x L-glutamine [2mM, GIBCO, cat. no. 25030-149]). Samples were then aseptically and finely diced using sterile scissors (Figure 4.2), vigorously vortexed and clarified (2,000g for 10 minutes at 4°C). The supernatant was then ready to be pipetted onto cultured cells.
Figure 4.2 Left: A sample of lung being diced with sterile scissors in a petri dish. Right: A diced sample of organ in virus isolation media prior to clarification.

Viper heart cells (VH2, ATCC CCL-140) (Figure 4.3), or rarely Vero cells (ATCC CCL-81) (Figure 4.4), were grown in either 25cm$^2$ flasks or 6-well plates (9.6cm$^2$/well) at 30°C and 5% CO$_2$ using cell culture maintenance media (MEM supplemented with 5% FBS, 1x enrofloxacin [12.5µg/mL], 1x amphotericin B [2.5µg/mL] and 1x L-glutamine [2mM]). At 80%-100% confluency, the culture medium was removed, the cells were rinsed with 1x phosphate-buffered saline (PBS, 130mM NaCl, 7mM KH$_2$PO$_4$, 3mM Na$_2$HPO$_4$.2H$_2$O, pH 7.4) and 1mL (for 25cm$^2$ flasks) or 0.75mL (for 9.6cm$^2$ wells) of the clarified supernatant from the tissue suspension or swab, was added to the cells. Flasks and plates were left to incubate at room temperature for one hour. After the supernatant had been removed, the cells were rinsed several times with 1x PBS and then 5mL (for 25cm$^2$ flasks) or 3mL (for 9.6cm$^2$ wells) of virus isolation media was added. Flasks and wells were observed daily for cytopathic effects (CPE) using a polarising inverted microscope. Seven days after inoculation, flasks and wells were frozen and then left to thaw at room temperature. Media were clarified as above and a 0.75mL aliquot of supernatant was then used to replace the maintenance media of 75%-80% confluent cells growing in 6-well plates. Wells were left at room temperature for one hour and then 2.5mL of maintenance media was then added to the 0.75mL of media in each well. For some virus isolation attempts, this freeze-thawing passage was repeated once more.
Two strains of ferlaviruses, Fer-de-Lance virus (FDLV, ATCC VR-895) (Figure 4.3) and neotropical ophidain paramyxovirus (ATCC VR-1408) (Figure 4.4), served as positive controls for virus isolation and propagation experiments.

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3 These two viruses were imported from the American Type Culture Collection (ATCC) through the ATCC’s exclusive Australian distributor, Cryosite. Cryosite arranged the permits necessary to allow the importation of these viruses into Australia.
Figure 4.3 Uninfected Viper Heart cells (VH2) (above) and VH2 cells infected with Fer-de-Lance virus (bottom). Characteristic cytopathic effects (CPE) of ferlavirus can be seen as syncytial cell formation (red arrow pointing to cluster of nuclei in the centre of a syncytial cell) and detachment of the monolayer (blue arrow pointing to area of flask where cells were once attached).
Figure 4.4 Uninfected Vero cells (above) and Vero cells infected with neotropical ophidian paramyxovirus (bottom). Characteristic cytopathic effects (CPE) of ferlaviruses can be seen as small syncytial cell formation (two examples are outlined in red).
Viral titre was determined using the Reed-Muench method as described by Mahy and Kangro (1996). Briefly, quintuplicate samples were added to host cells that had been grown in 96-well microtitre plates. Samples were usually added to cells in ten-fold serial dilutions but for viruses with low titres, the experiment was performed using three-fold dilutions to improve the resolution of the assay (Figure 4.5). The mathematically-derived dilution that produced observable cytopathic effects (CPE) in fifty percent of wells was then recorded. The inverse of this dilution was then expressed per millilitre to produce the tissue culture infectious dose (TCID<sub>50</sub>). Viral concentration was also estimated by the haemagglutination titre of the supernatant of infected culture flasks (Figure 4.6).

![Figure 4.5 Typical layout of 96-well plate for viral titre determination of both high-titre (left) and low-titre (right) viruses.](image)
Figure 4.6 The increasing haemagglutination titres of ferlaviruses seen during incubation in cell culture.

In addition to the methods described so far, the use of Ten Broeck (named after Thomas Ten Broeck) tissue grinders to make tissue suspensions suitable for virus isolation, and the establishment of a primary reptilian cell line to isolate viruses into, were also investigated.

Ten Broeck tissue grinders have successfully been used by others for the isolation of ferlaviruses from organ samples (Jacobson et al., 1980b; Jacobson et al., 1981; Jacobson et al., 1992). They were not used routinely in this study for three main reasons: the adherence of tissue to the glass surfaces made it difficult to separate supernatant from tissue, they were messy and therefore more likely to attract contamination and, for lung tissues, would only compress the organ sample without macerating it (Figure 4.7).
At the beginning of these studies, Vero cells were available for virus isolation but the successful isolation of reptilian paramyxoviruses using this mammalian cell line is limited to only two reports (Potgieter et al., 1987; Nogueira et al., 2002). A reptilian cell line would expand the number of substrates available to this project for virus isolation (see section 2.3.1 – Virus Isolation, particularly Table 2.7, for a review of this subject). Although an established reptilian cell line (VH2) was eventually imported from the European Collection of Cell Cultures (ECACC, Cat. No. 90102539), prior to this, attempts were made to establish a primary cell line of reptilian origin. A reptilian cell line that could be quickly developed in-house would avoid the considerable expense to import a cell line from Europe that required a CITES (Convention on International Trade in Endangered Species) permit. To establish a primary reptilian cell line, the explant method described by Clark et al. (1967; 1970; 1973) was used, which has produced three important cell lines: terrapene heart cells (TH-1, ATCC-CCL 50), viper heart cells (VH2,  

4 This cell line was imported through the commercial company Sigma. Sigma arranged the permits necessary to import this cell line into Australia.
ATCC CCL-140) and iguana heart cells (IgH2, ATCC-CCL 108). A large number of reptilian viruses have been isolated using these three reptilian cell lines (Jacobson, 2007).

Briefly, organs were salvaged from reptiles that had been euthanased at a veterinary hospital or from reptiles that had died recently due to road trauma. Animals that had displayed antemortem neurorespiratory signs were excluded. Two main considerations dictated what organs would be retrieved from these animals. Firstly, this project would involve the analysis of samples that had been obtained from snakes that died with neurorespiratory disease, so samples of lung and brain were collected. Secondly, TH-1, VH2 and IgH2 are all from heart explants and so the heart became the third priority sample. Figure 4.8 outlines the series of steps to establish a primary reptilian cell line. Organ samples were recovered aseptically, using the methods described previously for virus isolation. Organs were then cut into small pieces (approximately 1-3mm³) and explanted into 25cm² tissue culture flasks. A small amount of cell culture media (1mL) was added and the explants were incubated at 30°C and 5% CO₂. Flasks were examined daily and, one to three days later, an additional 1mL of media was added being careful not to dislodge the explants. This process was repeated until the total volume of culture media was 5mL.
Figure 4.8 Establishment of a primary cell line using an explant method. The organ (heart) is collected aseptically, minced, suspended in a small volume of cell culture media and then seeded into a culture flask.
In most cases, explants adhered to the culture flask but often cells did not propagate from the explants. Only in a few cases was significant cell growth seen and only in one case, a lung explant from a tiger snake (*Notechis scutatus*) (Figure 4.9), did the monolayer reach confluency (Figure 4.10). But even these cells could not be passaged. The cells remained adhered despite exposure to trypsin and so a cell scraper was used to dislodge the cells from the culture flask. Only a small percentage of these dislodged cells adhered to a new culture flask and after two weeks, the cell population had remained relatively unchanged. After several months and many attempts, the decision was made to abandon the goal of establishing a primary reptilian cell line. Instead, a reptilian cell line (VH2) was imported from overseas.
Figure 4.9 Attempt to establish a primary reptilian cell line using a sample of lung from a tiger snake (*Notechis scutatus*). Eight days after explantation. **Top:** Cells can be seen propagating around the explant. Scale bar represents 1mm. **Bottom:** Cell morphology eight days after explantation. Scale bar represents 100µm.
Figure 4.10 Attempt to establish a primary reptilian cell line using a sample of lung from a tiger snake (*Notechis scutatus*). 14 days after explantation. The flask has reached confluency. These cells could not be passaged. Scale bar represents 1mm.

4.2.2 Embryonated chicken eggs

Cell culture has been the main substrate used for the isolation of reptilian paramyxoviruses (Table 2.7). Only two reports describe attempts to isolate reptilian paramyxoviruses into embryonated vertebrate eggs. Clark *et al.* (1979) successfully isolated Fer-de-Lance virus using embryonated snake eggs and Manvell *et al.* (2000) was unable to isolate a ferlavirus into embryonated chicken eggs. In the studies presented in this thesis, embryonated chicken eggs were used mainly for the propagation of viruses that were already established in cell culture. The positive control ferlaviruses, neotropical ophidian paramyxovirus (ATCC VR-1408) and Fer-de-Lance virus (FDLV, ATCC VR-895), were propagated after they had been adapted to this new substrate.
8- to 12-day-old specific pathogen free (SPF) embryonated chicken eggs were kindly donated by the Department of Agriculture and Food Western Australia (DAFWA). The relevant anatomy of an embryonated chicken egg is illustrated in Figure 4.11. The series of steps involved in propagating viruses in embryonated chicken eggs are depicted in Figure 4.12. Eggs were candled to ensure the embryo was viable. This was defined by a rich supply of blood vessels (Figure 4.12) or movement of the embryo. The surface of an egg was then sprayed with 70% (v/v) ethanol, which was left to evaporate. Next, an engraver weakened a point on the surface of the egg that was approximately 30 degrees from the top of the egg. Using a 23G 1½ inch needle attached to a 1mL syringe, a 0.2mL aliquot of virus stock was inoculated into each allantoic cavity by inserting the needle through the weakened area of the egg to a depth of about 25mm. A small amount of air trailed the inoculum to accommodate for the needle’s dead space. Molten candle wax was used to seal the hole in the egg. Eggs were then left to incubate at virus permissive temperature (30°C). After incubation (3-7 days), any surviving embryos were euthanased by placing the eggs into a refrigerator for several hours. The surface of an egg was then sprayed with 70% alcohol and sterile scissors were used to aseptically dissect away the top of the egg to expose its contents. The egg was tilted slightly and a 1mL pipette tip was used to move the embryo against gravity to allow the allantoic fluid to pool on one side of the egg. A second 1mL pipette tip was used to collect the allantoic fluid being careful not to break the yolk sac. Approximately 3-9mL was obtained from each egg. The allantoic fluid was clarified (2,000g for 10 minutes at 4°C) to remove organic debris and then haemagglutination assays were used (section 4.3 - Haemagglutination) to quantify the amount of virus.

It was found that cell culture-adapted ferlaviruses required at least one passage in embryonated chicken eggs before significant increases in viral concentration were seen and inoculating the allantoic cavity with 0.2mL of virus stock seemed to produce higher concentrations of virus than inoculums of 1mL or 0.5mL (Figure 4.13). However, the variation
in virus concentration between the allantoic cavities of different embryonated chicken eggs that had been inoculated with the same amount of virus, showed a variation that was larger than the difference between the various inoculum volumes (Figure 4.14).

**Figure 4.11** Anatomy of a 10-day-old embryonated chicken egg showing the embryo, the air sac and the extra-embryonic membranes (1998).
Figure 4.12 Using embryonated specific pathogen free (SPF) chicken eggs to propagate ferlaviruses. The egg is candled to assess the viability of the embryo. The air sac can be seen at the top of the egg (green arrow). An engraver is used to weaken an area of the egg. Virus stock is injected into the allantoic space. Molten candle wax is used to seal the plug (red arrows) before eggs are left to incubate at a viral permissive temperature. After incubation, the top of the egg is removed to gain access to the allantoic fluid. The yolk sac is still prominent (purple arrow).
Figure 4.13 Haemagglutination titres of ferlavirus after incubation in embryonated chicken eggs. Significant haemagglutination activity cannot be detected in any of four eggs inoculated with cell culture-adapted ferlavirus after five days of incubation (above). The allantoic fluid from all four eggs was collected and pooled and then three different volumes were inoculated into four more eggs. The relationship between haemagglutination titre and the volume that is inoculated into the allantoic cavity of embryonated chicken eggs was recorded (bottom).

Figure 4.14 Haemagglutination titres of two strains of ferlavirus (neotropical ophidian paramyxovirus and Fer-de-Lance virus) grown in the allantoic cavities of six embryonated chicken eggs. The aliquots of each strain of virus that were inoculated into each egg were identical.
4.3 Haemagglutination Activity

Haemagglutination assays were performed on various sample types, each sample needing to be processed by a specific method (Figure 4.15). Oral and cloacal swabs were submitted in Hartmann’s solution, isotonic saline or viral transport medium (= cell culture medium). Swabs were vigorously vortexed in their media before aliquots were collected. To test cell culture flasks, either an aliquot of supernatant was taken without any further processing or the entire flask was frozen and then left to thaw at room temperature. If the latter, the thawed contents of the flask were clarified at 2,000g for ten minutes at 4°C to pellet cellular debris. Aliquots of this clarified medium were then collected. Prior to the haemagglutination assay, the clarified contents of some frozen-and-thawed flasks were concentrated by either polyethylene glycol (PEG) precipitation or ultracentrifugation.

For PEG precipitation, 10mL of clarified supernatant was made up to 100mL using phosphate buffered saline (PBS). Sodium chloride (NaCl) and PEG (MW ~6,000) were added to final concentrations of 2.2% (w/v) and 6% (w/v) respectively and the solution stirred continuously with magnetic stirrers for two hours at 4°C. The solution was then clarified at 2,000g for 20 minutes at 4°C. Supernatant was removed to reduce the volume by a factor of 100. Sodium acetate (50mM, pH 4.8) was then added to create a volume of 10.4mL. The solution was stirred again for 1 hour at 4°C and then clarified as before. This time, the supernatant was retained and the pellet was discarded. The supernatant was then poured onto a frozen 2.6mL 20% (w/v, dissolved in water) sucrose cushion in a 16mm x 76mm Beckman® Ultra-Clear tube and then ultracentrifuged at 148,000g for two hours at 4°C. Supernatant was discarded and the pellet was resuspended in 100µL of PBS.
Whole blood was prepared for use in a haemagglutination assay using the methods reported by Raidal et al. (1993). Whole blood was collected from the jugular vein of either a guinea pig or a chicken and then mixed with an equal volume of Alsever’s solution (0.42% [w/v] sodium chloride, 2.05% [w/v] glucose, 0.8% [w/v] trisodium citrate, 0.055% [w/v] citric acid monohydrate, pH 7.4). Alsever’s solution is both an anticoagulant and a blood preservative. Erythrocytes were then carefully washed. The whole blood-Alsever’s solution mixture was centrifuged at 300g for 10 minutes at 4°C, the supernatant was discarded and the erythrocyte pellet was then resuspended in phosphate-buffered saline (PBS). This process was repeated until the supernatant was clear. Pelleted erythrocytes were then added to PBS to create a working solution with a concentration of 0.5% (v/v) e.g. 65µL of erythrocytes to 13mL of PBS.

With the samples processed and the erythrocytes prepared, the haemagglutination assay was then set up (Raidal et al., 1993). Samples and positive and negative controls were each assigned to a row of a 96-well microtitre plate (Figure 4.16). Next, 50µL of PBS was added to all the wells in each row. 50µL of processed sample, or control, was then added to the well in the
first column and mixed with the 50µL of PBS already there. 50µL of the 100µL in this first well was then mixed with the 50µL of PBS in the second well. This was repeated for each well in the row creating doubling dilutions of the sample from the first well (1:2) to the twelfth well (1:4,096). The 50µL of surplus from the twelfth well was discarded. 50µL of washed chicken or guinea pig erythrocytes (0.5% v/v in PBS) were then added. The plate was then left to incubate for approximately one to three hours, at either room temperature or 4°C, to allow the erythrocytes to settle at the bottom of the well. Haemagglutination titres were read as the reciprocals of the greatest dilutions that resulted in complete haemagglutination (Figure 4.16). Two strains of ferlavirus, Fer-de-Lance virus (FDLV, ATCC VR-895) and neotropical ophidian paramyxovirus (nOPMV, ATCC VR-1408), served as positive controls for haemagglutination assays (Figure 4.16) and for PEG-precipitation experiments (Figure 4.17). Prior to the importation of ferlavirus, attenuated human influenza virus was used as a positive control for haemagglutination assays.

Figure 4.16 The first three rows of a microtitre plate showing a typical layout of positive and negative controls for a haemagglutination assay.
Figure 4.17 Concentration of positive control ferlavivirus by polyethylene-glycol precipitation (PEG-P). Ferde-Lance virus (FDLV) and neotropical ophidian paramyxovirus (nOPMV) were used as controls. Negative control was performed on a second plate and performed as expected.

4.4 Haemadsorption

Haemadsorption was assessed using the methods outlined by Mahy and Kangro (1996). Briefly, culture media was removed from a flask and the cell monolayer was rinsed with PBS. Chicken or guinea pig erythrocytes 0.5% (v/v in PBS) were then added to the flasks to cover the monolayer. Flasks were then left to incubate at room temperature for 30 minutes and then carefully examined while gently agitating the flask to look for firmly attached erythrocytes. Unattached erythrocytes were decanted and the monolayer examined again during gentle agitation. Finally, the monolayer was gently rinsed with PBS and examined once more. Uninfected viper heart cells (VH2) and VH2 cells infected with ferlavirus served as negative and positive controls respectively for this assay.

4.5 Haemagglutination Inhibition

Testing for anti-ferlavirus antibodies was performed using haemagglutination inhibition (HI). Samples were submitted as plasma, serum or as whole blood dried onto a disc of filter paper (= dried blood spots = DBS) (Whatman number 3 filter paper, Whatman International Ltd.,
Maidstone, England). For plasma or serum, the methods reported by Jacobson et al. (1992) were followed. Some “serum” samples that had been submitted frozen and then allowed to thaw contained variable amounts of haemolysis (Figure 4.18) and fibrin clots (Figure 4.19) due to incomplete clotting followed by inadequate separation of the supernatant from the cellular pellet. For these samples, the “serum” was centrifuged at 580g for 10 minutes at room temperature to pellet cellular debris, and a wooden applicator was used to remove any clots from the sample.

300µL aliquots of serum or plasma were then heated at 56°C for 30 minutes to inactivate heat-labile non-specific inhibitors of haemagglutination. Non-specific haemagglutinators were removed by haemadsorbing the serum aliquots to 10µL of pelleted chicken or guinea pig erythrocytes during an overnight incubation at 4°C (Figure 4.20).

![Figure 4.18](image) Frozen-thawed samples submitted as “serum” containing decreasing amounts of haemolysis from left to right. Good quality erythrocyte-free sample on far right.
Figure 4.19 Blood clots (blue arrow) and haemolysis-free fibrin clots (green arrow) that were removed from “serum” samples.

Figure 4.20 Haemadsorbing serum samples to remove non-specific haemagglutinators. Normal settling of erythrocytes in a sample free of haemagglutinators (left) contrasted to a haemagglutinated sample, rich in haemagglutinators (right).
As for the haemagglutination assay mentioned earlier, rows were assigned to controls or samples, and then 50µL of PBS was added to each well. Processed serum (or plasma) was then added to the first well and then pipetted across the row to create two-fold dilutions (1:2 to 1:4,096 [for 12 columns] or 1:256 [for 8 columns]). 50µL (titrated to 4-8 haemagglutination units) aliquots of neotropical ophidian paramyxovirus (ATCC VR-1408) were then added to each well. Next, the mixture of serum/plasma and virus was left for one hour at room temperature. Inhibition of viral haemagglutination was measured by the addition of 50µL of 0.5% (v/v) chicken erythrocytes to each well. HI titres were read as the greatest dilution that completely inhibited haemagglutination (Figure 4.21). PBS was used as a negative serum/plasma control and serum from a western diamondback rattlesnake (Crotalus atrox) with an HI titre of >256 / 50µL, kindly donated by Dr Rachel Marschang (University of Hohenheim, Germany), served as a positive control.

Figure 4.21 Controls for a haemagglutination inhibition assay. Top row: Positive control consisting of serum from a western diamondback rattlesnake (Crotalus atrox) with previous exposure to ferlavirus. HI titre = 16/50µL. Middle row: Two-fold serial dilutions of virus (neotropical ophidian paramyxovirus [ATCC VR-1408]) to determine the number of haemagglutinating units in each 50µL aliquot. HA titre = 4/50µL. Bottom row: PBS negative control. HI titre <2/50µL.

4.5.1 Eluting Anti-ferlaviral Antibodies from Filter Paper

For dried blood spots (DBS), a short experiment was designed to compare four different methods of antibody elution. A standard hole-punch was used to obtain a sample from each disc of filter paper (Figure 4.22). 400µL from two positive control serum samples (HI titres of >256 / 50µL) was added to two 38.5cm² discs of filter paper (keeping each serum sample separate). For the first elution method, a hole punch of filter paper from each positive control
sample was added to the first eluant (0.3% [v/v] Tween 20, 0.1% [w/v] sodium azide dissolved in phosphate-buffered saline [PBS]) and then sonicated in a water bath (Ultrasonicator FX-10, Unisonics, Sydney) for 15 minutes (Jafri et al., 1998). For method two, hole punch samples were left overnight at 4°C in the second eluant (5% [w/v] skim milk powder and 5% [v/v] Tween 20 dissolved in water) (Helfand et al., 2001). For method three, hole punch samples were added to the third eluant (0.05% [v/v] Tween 20, 1% [w/v] bovine serum albumin [BSA], 0.5% [w/v] gelatin dissolved in [PBS]) and mixed in a Thermomixer (Eppendorf Asia-Pacific) at 1,200rpm for two hours at room temperature before the eluant was passed through a 0.45µm filter (Yu et al., 2007). For the last method, hole punch samples were added to PBS and left at 4°C overnight.

![Figure 4.22](image)

**Figure 4.22** Whole blood from a diamond python (*Morelia spilota spilota*) dried onto a disc of Whatman filter paper. A hole punch is used to obtain a sample of blood from the filter paper. Ruler = 1cm major increments and 1mm minor increments.

Each eluted sample was then tested for anti-ferlaviral antibodies using both guinea pig and chicken erythrocytes as described above (**Figure 4.23**). The second and third methods appeared to perform better than a simple elution into PBS (the fourth method) but overall, only small differences were seen between the four different elution methods.
Figure 4.23 Comparison of four methods to elute anti-ferlaviral antibodies from filter paper. Each method was tested using two different positive controls and two different erythrocytes (RBCs) (chicken and guinea pig). Haemagglutination inhibition (HI) titres were recorded one hour after adding the erythrocytes to each well but photos were taken several hours later. As such, it is difficult to determine the HI titre in some rows from this image and so angled double yellow lines have been added after the well that showed complete inhibition of haemagglutination. Two double lines are used when it was difficult to determine the titre.

This short experiment provides evidence that at least four methods are able to elute anti-ferlaviral antibodies from snake serum that had previously been dried onto filter paper, but it does not show the number of anti-ferlaviral antibodies that were eluted as a percentage of those that were dried onto the filter paper. This “elution efficiency” should be investigated in future studies. In a study by Helfand et al. (2001), it was shown that there was concordance in the titres of anti-measles virus antibodies that were eluted off filter paper compared to those
in serum. Similarly, Jafri et al. (1998) showed there was no significant decrease in the sensitivity of dried blood spot samples compared to serum samples during a serosurvey of cysticercosis. Drying whole blood onto filter paper allows these samples to be transported and stored under conditions that are less stringent than those needed for serum samples (McDade et al., 2007). Only a small amount of whole blood is needed to create a dried blood spot that can be tested for the presence of antibodies and this will benefit reptile studies where animals can be small and/or difficult to collect larger volumes of blood from. So utilising dried blood spots for reptilian serodiagnosics could prove worthwhile.

4.5.2 Concentrating Anti-ferlaviral Antibodies by Ultrafiltration

In another short experiment, the ability to concentrate anti-ferlaviral antibodies by ultrafiltration was tested. Ultrafiltration is used to concentrate macromolecules in solutions by filtering and retaining molecules above a certain nominal molecular weight limit (NMWL e.g. 50,000 Daltons [Da]) using centrifugation. The flow-through is discarded and the retained molecules are then extracted by inverting the filter prior to a second centrifugation. In this experiment, positive control serum (known to contain anti-ferlaviral antibodies) was concentrated using a YM-100 Microcon ultrafilter (Millipore, Massachusetts, NMWL = 100kDa).

In snakes, a low-molecular-weight immunoglobulin (Ig) with a sedimentation coefficient of 7-9 svedbergs (S) is likely to be equivalent to avian IgY and mammalian IgG and a high-molecular-weight immunoglobulin (19.6-20.5S) is likely to be equivalent to mammalian and avian IgM (Origgi, 2007). Avian IgY and mammalian IgG have molecular masses of approximately 180kDa (Origgi, 2007; Tizzard, 2009) and avian and mammalian IgM have molecular masses of approximately 900kDa (Tizzard, 2009). This means that the 100kDa NMWL of the YM-100 Microcon ultrafilter would be likely to retain the snake equivalents of IgG and IgM.

Two 500µL aliquots of positive control serum were placed on top of two YM-100 Microcon ultrafilters which were then placed into microcentrifuge tubes and centrifuged at 14,000g for 12 minutes at room temperature. The pass-through that collected in the microcentrifuge tube
was kept. The ultrafilters were then inverted and placed into new microcentrifuge tubes to collect the eluate. The volumes of the eluants were then adjusted to create two concentrations: 16-fold and 22-fold. These two concentrated samples, along with the pass-through were then tested for the presence of anti-ferlaviral antibodies by haemagglutination inhibition (HI) as described earlier (Figure 4.24).

Figure 4.24 Detection of anti-ferlaviral antibodies by haemagglutination inhibition after concentration using ultrafiltration. Positive control material was concentrated by factors of ~16 and ~22. The flow-through was also tested to ensure that no antibodies could be detected by this assay.

This short experiment showed that anti-ferlaviral antibodies could be concentrated using ultrafiltration. By utilising this method, lower quantities of anti-ferlaviral antibodies could become detectable in an HI assay.

4.5.3 Unusual Clumping of Erythrocytes

Unusual clumping of red cells was seen in some haemagglutination (HA) and haemagglutination inhibition (HI) assays (Figure 4.25). To try and determine the cause, several of the steps of an HI assay were investigated (Table 4.1).
Only one of the methods in Table 4.1 reduced the incidence of abnormal erythrocyte clumping (data not shown). April Childress from the University of Florida (the largest reptilian diagnostic laboratory in America) made the suggestion that the working suspension of erythrocytes (0.5% v/v) should be vortexed prior to use. This small modification to the procedure provided far more reliable results (Figure 4.26).
### Haemagglutination Inhibition Assay

#### Erythrocyte Processing
- Adding 0.1% (w/v) bovine serum albumin (BSA) to the PBS that is used as a diluent for the erythrocytes. This is believed to reduce red cell autoagglutination (Jacobson and Origgi, 2007)
- Passing the PBS (red cell diluent) through a 0.45µm syringe-tip filter to remove non-filterable bacterial and fungal elements from the diluent
- Ensuring that the red cells used in the assay were fresh (less than a few hours old). It has been reported that the whole blood of chickens can be stored in an equal volume of Alsever’s solution at 4°C for up to two weeks prior to use in HA and HI assays for psittacine beak and feather disease (Raidal et al., 1993)
- Use a commercial Alsever’s solution (Sigma) rather than one made “in-house”
- Using erythrocytes sourced from guinea pigs rather than chickens
- Increase number of erythrocyte washes from three to five
- Vortexing the 0.5% (v/v) erythrocyte suspension prior to use (April Childress, University of Florida, personal communication)

#### Antigen Source
- Using cell culture derived antigen rather than antigen from the allantoic fluid of embryonated chicken eggs. Normal allantoic fluid of chickens has been shown to contain haemagglutination inhibitors of influenza virus and the GDVII strain of mouse encephalomyelitis virus (Tamm and Tyrrell, 1954)
- Using original antigen stocks (from ATCC) rather than antigen that has been propagated “in-house”
- Increased the concentration of antigen used in each well from 4 HA / 50µL to 8 HA / 50µL
- Clarify antigen immediately prior to use

#### Sample Processing
- Use fresh plasma/serum samples rather than samples eluted off filter paper
- Omit the haemadsorption of the serum/plasma
- Omit the heat inactivation (removes heat labile non-specific inhibitors of haemagglutination) of the serum/plasma
- Use kaolin (Raidal et al., 1993), receptor-destroying enzyme (RDE, Denka Seiken, Tokyo, Japan), dextran sulfate (Liebhaber, 1970; Monath et al., 1970), trypsin-periodate (Boliar et al., 2006) or heparin-MnCl₂ (Liebhaber, 1970) rather than heat to remove non-specific inhibitors of haemagglutination

#### Antigen-antibody Reaction
- Incubating the virus solution with the antibody sample at both 4°C and at room temperature

#### Unbound antigen-erythrocyte Reaction
- After the addition of red cells to detect the presence (or inhibition) of haemagglutination, allowing the incubation to occur at both 4°C and at room temperature

| Table 4.1 | Steps of a haemagglutination inhibition assay that were altered to determine the cause of irregular erythrocyte clumping. |  |
Figure 4.26 Comparison of two methods of erythrocyte preparation. The method used early in these studies ("old method") did not involve vortexing the working suspension of erythrocytes whereas the later method ("new method") did. Cleaner haemagglutination can be seen with the new method. PBS = phosphate-buffered saline.

The impact that haemolysis and blood clotting had on a ferlaviral haemagglutination inhibition assay was assessed. Two 50µL aliquots of positive control serum (known to contain detectable levels of anti-ferlaviral antibodies) from a western diamondback rattlesnake (*Crotalus atrox*) were diluted three-fold with two 100µL aliquots of fresh whole blood from a black-headed python (*Aspidites melanocephalus*). The first 150µL mixture was frozen and allowed to thaw. After centrifugation (500g x 5min), the supernatant was collected. This sample was to emulate a “serum” sample with significant haemolysis that still contained clotting factors. The second 150µL mixture was centrifuged immediately to pellet the erythrocytes and the supernatant was collected. This sample was to emulate a “serum” sample that still contained clotting factors. Both of these “serum” samples formed clots that were removed with wooden applicators as described above. To control this experiment, an aliquot of fresh whole blood from the black-headed python was added to lithium heparin, anticoagulated and centrifuged.
before the supernatant (plasma) was collected. A second aliquot of fresh whole blood was left to clot and then centrifuged before the supernatant (serum) was collected. Samples were then tested by HI for the presence of anti-ferlaviral antibodies using the methods described earlier (Figure 4.27). Although this experiment was limited to only one positive control sample spiked into the fresh whole blood of only one snake and no further replicates were performed, the experiment was able to demonstrate that the presence of a clot, and its subsequent removal with a wooden applicator, did not prevent the detection of anti-ferlaviral antibodies. The sample in this experiment required several hours to fully clot. The remarkable contrast between the ex vivo clotting times of mammalian blood and reptilian blood might explain why the University of Florida (http://www.vetmed.ufl.edu/college/departments/sacs/documents/HAIProtocol.pdf) requires plasma, rather than serum samples for their haemagglutination inhibition assay. As expected, the presence of haemolysis obscured the observer’s ability to detect haemagglutination inhibition but did not appear to significantly affect the assay in other ways.

![Figure 4.27](image_url) Impact that haemolysis and blood clots have on haemagglutination inhibition titres. Hyperimmune serum served as a positive control (HI titre 16/50µL, row 1) and was spiked into healthy snake blood (HI titre <2/50µL, rows 3 and 4) that contained clots (denoted as “serum”) with (row 5, HI titre 4/50µL) or without (row 6, HI titre 4-8/50µL) significant amounts of haemolysis.
4.6 Neuraminidase Activity

In 1996, Richter et al. described the successful adaptation of the methods reported by Yolken et al. (1980) to detect neuraminidase activity in three ferlaviral isolates. These methods were applied to the viral isolates used in this study. Using flat-bottom microtitre plates, an 80µL sample of cell culture supernatant or allantoic fluid was mixed with an equal volume of neuraminidase substrate: 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (Sigma, Cat. No. M8639) dissolved in a phosphate buffer solution (0.1M phosphate buffer [8mM Na₂HPO₄·2H₂O, 92mM NaH₂PO₄·2H₂O], 1mM CaCl₂, pH 5.8) to a final concentration of 0.1mM. The mixture was left to incubate at 30°C. After excitation at 355nm, the emission at 480nm was quantified using a fluorometer (Titertek® Fluorskan, EFLABoy, Finland) at various times after the substrate had been added to the viral solution (e.g. 30 minutes, 90 minutes, 3 hours and 24 hours). Attenuated human influenza virus (kindly donated by Dr Cassie Berry) and ferlavirus (ATCC VR-1408) were used as positive controls and media that the viruses were grown in (allantoic fluid or cell culture supernatant) were used as negative controls.

To investigate the robustness of this assay, different diluents, incubation times and sample dilution rates were assessed (Table 4.2). Although the virus permissive temperature of human influenza virus is 37°C, the virus was incubated with the neuraminidase substrate at 30°C. This was done to mimic the incubation temperature that would be used for any reptilian isolates.
### Table 4.2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Emission at 480nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment One (effect of diluent)</strong></td>
<td></td>
</tr>
<tr>
<td>Incubation time</td>
<td>1hr</td>
</tr>
<tr>
<td>hIV – phosphate buffer</td>
<td>257</td>
</tr>
<tr>
<td>hIV – PBS</td>
<td>237</td>
</tr>
<tr>
<td>hIV – cell culture medium</td>
<td>197</td>
</tr>
<tr>
<td>Uninfected phosphate buffer</td>
<td>-5</td>
</tr>
<tr>
<td>Uninfected PBS</td>
<td>-2</td>
</tr>
<tr>
<td>Uninfected cell culture medium</td>
<td>-11</td>
</tr>
<tr>
<td><strong>Experiment Two (effect of sample dilution)</strong></td>
<td></td>
</tr>
<tr>
<td>Incubation time</td>
<td></td>
</tr>
<tr>
<td>hIV – 1 in 8 dilution</td>
<td></td>
</tr>
<tr>
<td>hIV – 1 in 16 dilution</td>
<td></td>
</tr>
<tr>
<td>hIV – 1 in 20 dilution</td>
<td></td>
</tr>
<tr>
<td>hIV – 1 in 25 dilution</td>
<td></td>
</tr>
<tr>
<td>hIV – 1 in 27 dilution</td>
<td></td>
</tr>
<tr>
<td>hIV – 1 in 40 dilution</td>
<td></td>
</tr>
<tr>
<td>hIV – 1 in 80 dilution</td>
<td></td>
</tr>
<tr>
<td>Uninfected allantoic fluid (five replicates)</td>
<td></td>
</tr>
</tbody>
</table>

The amount of neuraminidase (NA) activity of an attenuated human influenza virus (hIV, 512HA / 50µL) compared to various uninfected culture substrates. The NA substrate (2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid) is exposed to the virus for certain lengths of time to produce a fluorescent substrate. The emission at 480nm provides an estimate of NA activity. For experiment one, hIV was propagated in the allantoic cavity of embryonated chicken eggs and then NA activity was assessed after dilution in three different diluents. For experiment two, uninfected allantoic fluid was mixed with allantoic-derived hIV to seven dilution rates.

### 4.7 Electron Microscopy

Electron microscopy was used on a number of samples to search for viruses. Transmission electron microscopy (TEM) was used to search for viruses in cell culture media and infected cells while scanning electron microscopy (SEM) was used on occasion to visualise viruses on the surface of haemagglutinated chicken erythrocytes.

#### 4.7.1 Transmission Electron Microscopy

A variety of sample types were processed in a number of ways and then negatively stained with phosphotungstic acid before examination by transmission electron microscopy (TEM)
Clarification, polyethylene glycol (PEG) precipitation and ultracentrifugation were performed as previously described (section 4.3 – Haemagglutination). After sample processing, 2µL aliquots of each sample were absorbed onto formvar-coated copper mesh grids for ten minutes. Excess liquid was removed using a small piece of filter paper. The surface of the formvar-coated grid was then negatively stained for three minutes using a 2µL aliquot of phosphotungstic acid (PTA, 1% v/v in Tris-buffered water, pH 7.3). Excessive liquid was again removed with filter paper. Grids were examined using a Philips CM100 Biotwin transmission electron microscope (Philips/FEI Corporation, Eindhoven, The Netherlands). Attenuated human influenza virus (kindly donated by Dr Cassie Berry) (Figure 4.29) and Nelson Bay orthoreovirus (Figure 4.30) served as positive controls.
Figure 4.28 Transmission electron microscopic (TEM) examination of several different sample types. 
Top: Cell culture media and infected cells were processed in various ways for examination by TEM after negative staining. Bottom: Infected cells and histological sections (in this case, inclusion bodies in a section of snake brain) were positively stained prior to examination by TEM. PEG = polyethylene glycol.
Figure 4.29 Transmission electron microscopic view of two negatively stained human influenza virions (red arrows). The spiked envelope can be seen on the surface of each virion (a section of envelope has been bordered by two blue lines).

Figure 4.30 Transmission electron microscopic view of Nelson Bay orthoreovirus after negative staining. **Top:** Two intact virions. **Bottom Left:** Double capsid structure can be seen in this partially digested virion. **Bottom Right:** A typical empty shell.
Infected cells from cell culture and histological sections were positively stained prior to examination by TEM (Figure 4.28, bottom panel). For infected cells from cell culture, media was removed, the monolayer was rinsed with PBS and cells were then trypsinised and pelleted (580g x 10 minutes). Trypsin was removed and the cells were then resuspended in 5% glutaraldehyde (v/v in Sörensen’s phosphate buffer [12.9mM KH₂PO₄, 53.1mM Na₂HPO₄, pH 7.4]) and left for two hours at 4°C for fixation. Cells were pelleted, glutaraldehyde was removed and the cells were resuspended in Sörensen’s phosphate buffer to remove residual glutaraldehyde. Cells were again pelleted, supernatant removed, and cells were resuspended in a drop of molten agarose (2% w/v in water). Immediately afterwards, cells were pelleted at full speed on a bench top centrifuge (~20,000g x 1 minute). Cells were moved to a refrigerator and once the agarose had solidified (approximately 10 minutes), the agarose plug was carefully removed from its tube and the cell pellet was dissected away from the rest of the agarose. The agarose-encased and glutaraldehyde-fixed cell pellet was then processed before being positively stained.

First, the cell pellet was submerged in Dalton’s chrome osmic acid (1% w/v osmium tetroxide [OsO₄], 1% w/v K₂Cr₂O₇, 0.85% w/v NaCl, pH 7.2) for one hour at 4°C. The Dalton’s chrome osmic acid was decanted away and the sample was dehydrated. The sample was washed three times, each for two minutes, with each of four concentrations (v/v) of ethanol: 70%, 90%, 95% and 100%. The sample was then washed twice, each for two minutes, with 100% propylene oxide. After the propylene oxide had been removed, the sample was then infiltrated with a 60:40 mix of propylene oxide and EPON (24.6% w/v dodecenyl succinic anhydride hardener 964, 47.1% w/v Taab 812 resin, 28.2% w/v methyl nadic anhydride) and left to incubate at 4°C for one hour. The propylene oxide-EPON mixture was removed and EPON was used to cover the sample which was then left to incubate on a slow moving rotor (approximately 1-5 rpm) at room temperature for 12 hours. The EPON and sample were then transferred to an embedding capsule and the EPON resin was left to polymerise during a 24 hour incubation at 60°C.
the resin plugs were removed from the embedding capsules and semi-thin sections were cut (1µm) and stained with a toluidine blue solution (1% w/v toluidine blue and 0.5% w/v sodium tetraborate [borax]). Semi-thin sections were assessed by light microscopy for their suitability as ultra-thin sections (70-90nm). If the sample quality was adequate, several ultra-thin sections were then cut and placed onto copper grids. Ultra-thin sections were then ready for positive staining. Sections were incubated in a drop of saturated uranyl acetate for five to seven minutes. Uranyl acetate was cleared by submerging the sections in distilled water. After drying, the sections were stained with 0.1%-0.4% (w/v) lead citrate for four minutes, which was then washed away in distilled water. After drying, the grids containing the sections were coated in formvar before being examined by TEM.

For histological sections, areas of interest (usually inclusion bodies) were identified. Coverslips were then removed by soaking the slide in xylene at 60°C. The slide was then placed into fresh xylene at room temperature for five minutes. Next, the slide was transferred to a 50:50 solution of xylene and propylene oxide for five minutes, and then a solution of pure propylene oxide for a further five minutes. The slide was covered in a 25:75 solution of EPON and propylene oxide for one hour. An embedding capsule was filled with EPON and then placed over the area of interest of the slide and left to polymerise for 24 hours at 60°C. The slide was then attached to the embedding capsule and the slide and capsule were immersed in liquid nitrogen where the slide separated away from the capsule leaving the section embedded in the capsule’s EPON. Finally, ultra-thin sections were cut, placed onto copper mesh grids, positively stained as previously described, coated in formvar and viewed under TEM.

**4.7.2 Scanning Electron Microscopy**

Only haemagglutinated chicken erythrocytes were examined under a scanning electron microscope (SEM). Haemagglutinated erythrocytes were fixed in glutaraldehyde and then pelleted. After removing excess glutaraldehyde, the fixed erythrocytes were incubated in Dalton’s chrome osmic acid at 4°C for two hours. The sample was then washed in several
changes of 30% (v/v) ethanol until no more osmic acid washed away. Next, the sample was washed twice, each time for five to ten minutes, with each of five concentrations (v/v) of ethanol: 50%, 70%, 80%, 90% and 95%. After three ten-minute washes in 100% ethanol, the sample was then left on a specimen stub to air-dry causing the cells to adhere to the stub. The sample was then splattered (coated) with gold and then stored in a desiccator until it was viewed in a Philips XL 20 scanning electron microscope (Philips/FEI Corporation, Eindhoven, The Netherlands). Two viruses with high haemagglutination titres were used as positive controls: human influenza virus (2,048HA/50µL) and ferlavirus (256HA/50µL) (Figure 4.31).
4.8 Polymerase Chain Reaction

Many different sample types were processed for the detection of a range of viruses by polymerase chain reaction (PCR) (Figure 4.32). The objective of sample processing was to extract the nucleic acid (RNA and/or DNA) from a diverse range of sample types: cell culture, swabs, fresh tissue samples and formalin-fixed tissue samples.

Figure 4.32 Various sample types that were processed for the detection of a range of viruses by polymerase chain reaction
200-400µL aliquots of unprocessed media and frozen-thawed, clarified cell lysate were taken from infected and uninfected flasks. Total nucleic acid (RNA and DNA) was extracted from each using the MagMAX™ Viral RNA Isolation Kit (Ambion, Austin, Texas, cat. no. AM1939) according to the manufacturer’s protocols. For freeze-dried vaccines, lysis/binding solution from the Ambion extraction kit was added to the vaccine vial to dissolve the contents. From there, total nucleic acid extraction proceeded in accordance with the manufacturer’s recommendations.

For combined oral-cloacal swabs, a cotton-tipped applicator was pre-moistened in isotonic saline (or Hartmann’s solution) and then the inside of the snake’s mouth (especially the glottis) and cloaca were swabbed. Oral-only and cloacal-only swabs were also taken from a subset of the snakes. All swab tips were broken off into sterile containers, submerged in isotonic saline (or Hartmann’s solution). Containers that contained immersed swab tips were vigorously vortexed for at least 30 seconds and then a 200µL aliquot of the saline or Hartmann’s solution was used for total nucleic acid extraction using the Purelink™ Viral RNA/DNA Mini Kit (Invitrogen, Victoria, cat. no. 12280-050) according to the manufacturer’s instructions.

Fresh tissues were submitted in isotonic saline or Hartmann’s solution, while freshly-frozen tissues were submitted in small sterile containers without media. A small (1-3mm³) piece of tissue was collected from each organ sample and using the MELT™ Total Nucleic Acid Isolation System (Ambion, Texas, cat. no. AM1983), total nucleic acid was extracted from the sample in accordance with the manufacturer’s instructions. For formalin-fixed paraffin-embedded (FFPE) samples, sections (4-10µm) were cut with a fresh microtome blade that was wiped with xylene between sections. Total nucleic acid was recovered as per the methods described for fresh and freshly-frozen tissues, but with minor modification. Samples were first deparaffinised in two washes of xylene. Each wash consisted of adding 1mL of xylene to a microcentrifuge tube that contained the sections from each sample being processed. The tube was then vortexed and
incubated for three minutes at 50°C. After centrifugation at ~20,000g for two minutes, the supernatant was removed. This xylene wash was repeated once more and then the xylene was cleared with two washes of ethanol. An ethanol wash consisted of adding 1mL of 100% ethanol to the deparaffinised sections, vortexing the tube, pelleting the tissue at ~20,000g for two minutes and then removing the supernatant. Deparaffinised tissues were then digested overnight at 50°C in the digestion cocktail provided with the Ambion kit. After this extended digestion, the sample was treated identically to a fresh tissue that was being processed with the same extraction kit.

Total nucleic acid was always eluted into 30µL of elution buffer. For the detection of RNA viruses, the RNA in extracted total nucleic acid was reverse transcribed into complementary DNA (cDNA). For this, 13.5µL of total nucleic acid was added to 1µL of random hexamers (100ng/µL) (except some reovirus PCRs, see later) and 1µL of dNTPs (10mM) and incubated at 65°C for five minutes. 0.5µL of Superscript® III reverse transcriptase (200 units/µL, Invitrogen, Victoria, cat. no. 18080-044) and 4µL of 5x buffer were then added to make a final volume of 20µL which was then incubated at 25°C for five minutes, 45°C for 45 minutes and 70°C for 15 minutes. For PCR amplification, 1µM (final concentration) of each primer was added to 1µL of cDNA and then Platinum PCR Supermix® (Invitrogen, Victoria, cat. no. 11306-016) was used to bring the final reaction volume to 20µL.

Thermocycling always started with a two minute incubation at 94°C to remove anti-taq antibodies. Various cycling conditions followed that were dictated by the recommendations published for each particular primer set. Where primers were designed in house, denaturation was always at 94°C for 20 seconds. The annealing temperature was set at 3-5°C below the lowest melting temperature (Tm) of the primer set and incubation at this temperature lasted for 20-45 seconds. The extension temperature was always 72°C and incubation lasted for 20
seconds to one minute depending on the anticipated amplicon size. Each PCR continued for 40 cycles.

Several different primer sets were used for the detection of reoviruses (Table 4.5) and for all except those described by Landolfi et al. (2010) and Wellehan et al. (2009), random hexamers were used to reverse transcribe RNA into cDNA. When using the primer sets published by Landolfi et al. (2010) and Wellehan et al. (2009), both primers from the first round of the PCR were used as gene specific primers in the reverse transcription of RNA. Gene-specific primers were added (0.5µL of 30-80µM) to the RNA template along with dNTPs before the mixture was incubated at 94°C for two minutes, and then chilled rapidly to 4°C using ice water. After the addition of reverse transcriptase and its buffer, the solution was then incubated at 45°C for 45 minutes and 70°C for 15 minutes. Prior to the addition of the labile enzyme reverse transcriptase, RNA templates are often incubated at 60°C to remove secondary structure but in the case of double-stranded RNA (dsRNA) viruses like reoviruses, not only was a higher temperature used (94°C) to denature the dsRNA into single strands (Davis and Boyle, 1990) but gene specific primers that target both strands of the viral genome were used. When using the PCR primers reported by Landolfi et al. (2010) and Wellehan et al. (2009) on reovirus templates, specific amplicons could only be detected when using gene specific primers, and not random hexamers, to reverse transcribe the RNA.

The PCR primers that were tested on the nucleic acid that had been extracted from cell culture, vaccines and tissues, are listed in Tables 4.3 – 4.5. Primers were either designed in-house or carefully selected from published reports. For published primer sequences, primers with the demonstrated ability to detect a diverse range of viruses within a particular group were chosen. For primers designed in-house, primers were either designed manually or with the aid of computer software (Primer Premier®, Version 5, PREMIER Biosoft International). The investigative nature of this project meant that the identity of the virus (or viruses) was not
known, so primers that could theoretically detect all the members of a particular genus or family were designed. To do this, a conserved region of the viral genome was selected. The polymerase gene has been consistently chosen for ferlaviruses (Ahne et al., 1999b), paramyxoviruses (Tong et al., 2008), adenoviruses (Wellehan et al., 2004) and herpesviruses (VanDevanter et al., 1996), and so efforts were focussed on this gene. Next, sequences from several viruses in a particular group (e.g. ferlaviruses, orthoreoviruses) were downloaded from GenBank (National Center for Biotechnology Information, Bethesda, Maryland) and then aligned using ClustalW (Thompson et al., 1994). Nucleic acid sequences were aligned for closely related viruses (e.g. ferlaviruses) but amino acid sequences were aligned for more distantly related viruses (e.g. paramyxoviruses). Highly conserved areas were identified and primers were designed to anneal to these areas. Primers were chosen based on numerous criteria: minimal predicted hairpin, dimer and cross-dimer formation; weak annealing to other areas of the virus genome (false-binding); BLASTN (Basic Local Alignment Search Tool for Nucleotides, http://blast.ncbi.nlm.nih.gov/Blast.cgi) failed to find similar sequences in the GenBank database; and for primer pairs, the melting temperatures were similar for each primer.

For some PCRs, the stringency was lowered by decreasing the annealing temperature. The positive controls used for paramyxovirus, ferlavirus, reovirus, herpesvirus and adenovirus PCRs were canine distemper virus (Protech C3, Fort Dodge, Auckland), a neotropical strain of ferlavirus (ATCC VR-1408), Nelson Bay orthoreovirus (kindly donated by Professor Graham Wilcox), equine herpesvirus (G. Wilcox) and canine adenovirus type 2 (Protech C3) respectively.

PCR products were separated using 2% agarose gel (w/v dissolved in Tris-Acetate-EDTA buffer) electrophoresis at 80 Volts for 80 minutes and visualised using 0.005% SYBR® safe (v/v in the agarose-Tris-Acetate-EDTA gel; Invitrogen, Mulgrave, Victoria, cat. no. S33102) and a transilluminator (DR88M Dark Reader non-UV Transilluminator; Clare Chemical Research Inc.,
Dolores, Colorado). Bands of correct size were excised from the gel using a sterile scalpel blade, purified using the Purelink™ Quick Gel Extraction Kit (Invitrogen, Mulgrave, Victoria, cat. no. K210012) and then directly sequenced on an ABI 3730xl DNA Analyser (Applied Biosystems, Foster City, California). Primer sequences were excluded from the amplicons, which were then compared to the GenBank database (www.ncbi.nlm.nih.gov/Genbank/index.html).
### Table 4.3 Primers used for the detection of the polymerase gene (L) of paramyxoviruses, especially ferlaviruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genome region</th>
<th>Primers (5’ → 3’), amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paramyxoviridae</strong></td>
<td>Polymerase (L)</td>
<td>First round</td>
<td>Second round</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2 (TTATGAGATCTACAGAAAGGACA)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A51 (AGGAATTGGAGATGATT), 154bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A53 (CATAGTCTGAGGAAGGA), 107bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S4 (GATTATGAGATCTACAGAAAGGACA)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A55 (TGGGAGATAATGCTTTC), 274bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S7 (GGACAGGCGCTATTCC)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A56 (ATTCTTTCTTCTTCAGACTATA), 223bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>qS2 (GTATGCGAATCATGCTTGAGA), 157bp</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>qAS2 (CTGATGGAGAAATGCTTGAGAT), 109bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DegenParamyxoF (GGIGGKATWGAAGGWTWITGYCAAAAAMTRTGAG)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DegenParamyxoR (TKAYTGCWATTGMTTGATGCT), 566bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L5 (GCAGGAGTTTTCTTTCTTT)</td>
<td>L7 (TAGAGGCCTGTACTGCTGC), 566bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L6 (AGCCTCTTTTGTATGCTC), 627bp</td>
<td>L5 (CATCTTTGCAATTATAGCTG), 627bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAR-F1 (GAAAGGAGTACCAGAAAGGAG)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAR-R (GCTGAGTTACGIGCICCDAT), 662bp*</td>
<td>PAR-R, 584bp*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAR-F2 (GTTGCTTCAATGGTTCARGGNG)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAR-R, 584bp*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNE-F1 (GTGTAGGTAGIATGTTYGCN)</td>
<td>PNE-F2 (ACTGATCTIAGYAARTTYAAYCARG), 488bp*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNE-R (GCTTCCAAIITTTGGAGGATCAC), 264bp*</td>
<td>PNE-R, 264bp*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNE-F2 (ACTGATCTIAGYAARTTYAAYCARG)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNE-R, 264bp*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pmxF (TACTGCTTATATTGGGATAGA)</td>
<td>pmxR (CCTTCTACCTTTTCTTAGATA), 224bp*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pmxR (CTTCTACCTTTTCTTAGATA), 224bp*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PMV14610F (TTTGCNAAATGACNTACAAATGAG)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PMV15538R (GAAGGNTATTGGNCAGANATAGGAC), 744bp</td>
<td></td>
</tr>
</tbody>
</table>

- *Information not provided in original manuscript but estimated from binding sites in *Fer-de-Lance virus (FDLV), see Figure 6.9.
- #Bovine respiratory syncytial cell virus (BRSV), see Figure 6.9 or #human parainfluenza virus (GenBank accession number: FJ455842.2)
<table>
<thead>
<tr>
<th>Virus</th>
<th>Genome region</th>
<th>Primers (5’ → 3’), amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferlaviruses</td>
<td>Haemagglutinin - Neuraminidase (HN)</td>
<td>S2 (TATGGAGGACTGATGACACC)</td>
<td>Designed in-house</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS1 (GCACCCCGCTAATG), <strong>247bp</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AS3 (AGAAATACCCTATTAGTTGACTTACT), <strong>109bp</strong></td>
<td>Designed in-house</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HN1 (AAATCTGCAGTACCCTGGA)</td>
<td>(Ahne et al., 1999b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HN2 (AGATATCTGTGAAACTCTCTG), <strong>679bp</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HN3 (GACTCTACCCCCAGTTGG)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HN4 (TTCTATCCAGCTATTCTTAT), <strong>399bp</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.4** Primers used for the detection of the haemagglutinin-neuraminidase gene (HN protein) of ferlaviruses.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Genome region</th>
<th>Primers (5’ → 3’), amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Reoviridae</em></td>
<td>Polymerase (L1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>First round</strong></td>
<td><strong>Second round</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoS (AACAAACGACGATGATGAA)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoAS1 (ACCATTACCATCACACC), 137bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoA-F (GCAAGTAYCTYCAACTACGAA)</td>
<td>Single round only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoB-R (RTTRGCTNRTGRCRTNRTG), 324bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoA-F</td>
<td>ReoA-F, 324bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoC-R (NCCRTCTCNCCYGRANACRARTT)*, 447bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoA-F</td>
<td>ReoA-R, 447bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoA-F</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoD-R, 594bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoB-F</td>
<td>ReoB-R, 270bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoA-F</td>
<td>ReoB-R, 270bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ReoC-R, 123bp</td>
<td></td>
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<td></td>
<td></td>
<td>ReoD-R, 270bp</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>ReoC-R, 147bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1.rv5 (GCAAGTAYCTYCAACTACGAA)</td>
<td>L1.rv7 (GCTAGCCGATATCGGAATGCAG)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1.rv6 (GCAAGTAYCTYCAACTACGAA)</td>
<td>L1.rv7 (GCTAGCCGATATCGGAATGCAG)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1.rv5m (GCAAGTAYCTYCAACTACGAA)</td>
<td>L1.rv5m (GCAAGTAYCTYCAACTACGAA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1.rv6m (GCAAGTAYCTYCAACTACGAA)</td>
<td>L1.rv6m (GCAAGTAYCTYCAACTACGAA)</td>
</tr>
<tr>
<td>Primers Used</td>
<td>Sequence</td>
<td>Product Size</td>
<td>References</td>
</tr>
<tr>
<td>--------------</td>
<td>----------</td>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>σA capsid protein (S1, S2)</strong></td>
<td>1607F (CARMGNCNGSNCHMTATGATCC) 2608R (TAVAYRAAVGWCCSMHNGRTAYTG), 1,053bp</td>
<td>2090F (GGBTCMACNGCYACYTCBACYGAGCA) 2334R (CDATGTCRTAHWYCCANCRCRAA), 292bp</td>
<td>(Wellehan et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>1607F 2608R, 1,053bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1607F 2608R, 1,053bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1607F 2334R, 775bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2090F 2608R, 570bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2090F 2334R, 292bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2090F 2334R, 775bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2090F 2200R (CCRTCRTCWCCTCYGRTAYSCTART), 162bp</td>
<td></td>
<td>(Landolfi et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>PAF (ACTTCTTYTCTAGCTTCTCG) PAR (ATYAAWDCWCCGCTGCTTG), 598bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inner coat protein (S6)</strong></td>
<td>AQ1 (AWSCCKTAYTATGCTTTG), 443bp</td>
<td>Single round only</td>
<td>(Seng et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>AQ2 (TTRGAGACGAAAAAACNGACC), 443bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Herpesviridae Polymerase</strong></td>
<td>Forward 1: DFA (GAYTTYGCNAGYYTNTAYCC), 735bp</td>
<td>TGV (TGTAACTCGGTGTAYGGNTTYACNGGNGT), 232bp</td>
<td>(VanDevanter et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Forward 2: ILK (TCCTGGACAACGARNYSGCNMTNAA), 479bp</td>
<td>IYG (CACAGAGTCCTCCTCCNCTCCTADAT), 232bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse 1: KG1 (GTCTTGCTCACCAGNCTCNACNCCYTT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Adenoviridae Polymerase</strong></td>
<td>AdenopolRouter (GTGDCRAANSNHCRRCTABARNMRTT), ~533bp</td>
<td>AdenopolRouter (CCANCCBCDRTTRTGRNARNGTRA), ~533bp</td>
<td>(Wellehan et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>AdenopolFouter (TNGNNGGNGNMTGNTATCC), ~533bp</td>
<td>AdenopolFinner (GTNTWYGAHTHTYGGHATGC), ~320bp</td>
<td></td>
</tr>
</tbody>
</table>

*Table 4.5* Primers used for the detection of the genes that code for the polymerase, σA capsid and inner coat proteins of *Reoviridae*, orthoreoviruses and aquareoviruses respectively. Primers used for the detection of the polymerase genes of herpes- and adenoviruses are also included. R = A or G; Y = C or T; W = A or T; K = G or T; S = C or G; H = A, C or T; M = A or C; D = A, G or T; V = A, G or C; B = G, C or T; N = A, C, G or T. * First round PCR products were electrophoresed looking for appropriately-sized amplicons. # Amplicon size influenced by species of herpesvirus. Estimates provided here are for human herpesvirus 1 (GenBank number: JN555585.1).
4.9 In situ Hybridisation

In situ hybridisation (ISH) has been used for the sensitive detection of ferlaviral RNA from archival sections of formalin-fixed paraffin-embedded (FFPE) tissues (West et al., 2001; Sand et al., 2004). In both publications, DNA oligonucleotides were used as probes. West et al. (2001) used probes that had been designed for the detection of avian paramyxoviruses, while the probes published by Sand et al. (2004) were specifically designed for the detection of ferlaviruses. For the studies presented in this thesis, the oligonucleotide sequences reported by Sand et al. (2004) were used. In 2002, Jones described the steps needed to detect mRNA with digoxigenin-labelled DNA oligonucleotide probes and this protocol was followed. The process can be divided into four sections: probe labelling, sample preparation, hybridisation and detection.

One hundred picomoles of each DNA oligonucleotide was labelled at the 3’ end with digoxigenin using the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche, Mannheim, Germany, cat. no. 03 353 583 910), according to the manufacturer’s instructions. The final concentration of labelled probe was 100pmol/22µL (4.55µM, or approximately 20-40ng/µL, depending on the molecular weight of the particular oligonucleotide).

For sample preparation, six micron (6µm) sections were cut from FFPE tissues, placed onto silanised slides and left unstained. Sections were deparaffinised in two three-minute washes of xylene. Tissues were then hydrated after two one-minute washes in 100% and then 95% (v/v) ethanol that were followed by two three-minute immersions in pure water. Slides were then immersed in 2x standard saline citrate (SSC) buffer (300mM NaCl, 30mM trisodium citrate, pH 7) at 70°C for ten minutes. Following this, slides were placed into pure water for five minutes. Tissue sections were then covered with 100µL of a proteinase K solution (20µg/mL in Tris-buffered saline [50mM Tris, 150mM NaCl, pH 7.6]) and left to incubate at 37°C for one hour.

130 | P a g e
Slides were then twice immersed in pure water at 4°C for five minutes. Next, slides were transferred into 0.4% paraformaldehyde (w/v dissolved in 1 x phosphate-buffered saline [130mM NaCl, 7mM KH₂PO₄, 3mM Na₂HPO₄·2H₂O, pH 7.4]) and left to incubate at 4°C for 20 minutes before being immersed in pure water for five minutes.

For hybridisation, each section was first covered in 50µL of pre-hybridisation solution (600mM NaCl, 0.1% w/v sodium pyrophosphate, 0.2% w/v polyvinylpyrrolidone [MW 40,000], 0.2% w/v Ficoll [MW 40,000], EDTA 5mM, Tris-Cl 50mM, 10% w/v dextran sulphate, 31.5% v/v formamide, dissolved in water) and incubated at 37°C for one hour. After adding 50µL of hybridisation solution (0.5µL [approximately 10−20ng] of each probe made up to 50µL with pre-hybridisation solution) to each section, coverslips were applied and the sections were then left to incubate overnight at 37°C. The next day, the coverslips were removed and the hybridisation solution was rinsed with 2x SSC before slides were twice immersed in 2x SSC at 37°C for ten minutes.

For detection, sections were covered in 100µL of a 600-fold dilution of anti-digoxigenin alkaline phosphatase conjugate (Anti-Digoxigenin-AP Fab fragments, 150 units in 200µL, Roche, Mannheim, Germany, cat. no. 11 093 274 910, diluted in Tris-buffered saline) and left to incubate for 30 minutes. Sections were then rinsed in two five-minute washes of Tris-buffered saline. Next, sections were washed in alkaline phosphatase substrate buffer (100mM Tris-HCl, 100mM NaCl, 50mM MgCl₂) for five minutes. After covering slides in 200µL of an alkaline phosphatase substrate-levamisole solution (196µL of BM Purple AP Substrate, precipitating, Roche, Mannheim, Germany, cat. no. 11 442 074 001, and 4µL Levamisole® 32g/L, Western Stock Distributors, Perth, Australia), sections were left in the dark to incubate overnight. Slides were then examined for hybridisation before they were rinsed under running water for five minutes. Slides were exposed to a 30 second immersion in a bluing solution and
then stained with Brazilin for two minutes. Residual stain was cleared with pure water and a coverslip was mounted using Apathy’s mountant, an aqueous mountant.

Alkaline phosphatase activity is found in many different tissue types and levamisole has the ability to block *endogenous* alkaline phosphatase (except intestinal sources) while sparing the activity of *exogenous* alkaline phosphatase (Ponder and Wilkinson, 1981). To analyse the endogenous alkaline phosphatase activity in various snake tissues, a one-off bench-top experiment on duplicate sections was performed. A section consisting of snake brain, lung and liver was exposed to 200µL of alkaline phosphatase substrate solution containing levamisole (see above for solution compositions), while a second section from the same snake tissues was exposed to the substrate solution without levamisole. In the section stained in the absence of levamisole, non-specific staining of the liver was noted, particularly in areas of high fat content. There was a moderate amount of staining in the lung but none could be detected in the brain. In the presence of levamisole, very little staining could be seen. This was taken as meaning that the snake sections examined contained significant amounts of endogenous alkaline phosphatase activity that could be quenched with levamisole.

Primers were designed for the detection of 18S ribosomal RNA (rRNA) (18S rRNA antisense: 5’ AAACCATCCAATCGGTAGTAGC; 18S rRNA sense: 5’ CCAGTAAGTGGCGGTCACAAGC). Using these primers, 18S rRNA was detected by PCR in formalin-fixed paraffin-embedded (FFPE) snake tissue (data not shown). To control for the ISH methodology described, 100pmol of each primer was labelled with digoxigenin and used as a probe to hybridise to 18S rRNA in a variety of FFPE snake tissues. Snake sections known to be infected with ferlavirus were not available for examination.
Chapter 5 – Sample Examination Leading to the Isolation of a Virus Named BHP1-Lung

This chapter describes the snake samples that were sourced from around Australia and the tests that were performed on them that ultimately lead to the isolation of a virus, initially referred to as BHP1-Lung. The chapter is divided into four main sections. In the first section (5.1 – Sample Collection), samples are tabled according to type (e.g. swab, tissue, blood etc) and what tests were performed on them. Some of the tests were selected for their abilities to detect paramyxoviruses (e.g. polymerase chain reaction [PCR], haemagglutination and neuraminidase assays) while others were selected for their capabilities to detect a broader range of viruses (e.g. virus isolation and electron microscopy). The second section of this chapter (5.2 – Screening Samples for Viruses) describes the results of the sample testing. Eventually, a virus was isolated in cell culture and the third section of this chapter (5.3 – Laboratory Investigations of BHP1-Lung) describes the tests that were performed on this isolate to try and determine the family that it belongs to. The final section (5.4 - Chapter Summary) summarises the results of sample testing and the more specific testing that was performed on BHP1-Lung.

5.1 Sample Collection

From the period of May 2007 to October 2009 various snake samples were received from veterinarians for virus screening (Table 5.1). In total, 463 samples were collected from 192 snakes from 36 reptile collections from five Australian states or territories. Samples were received from all states and territories except Tasmania and the Australian Capital Territory (ACT). All samples were provided by veterinarians that worked with privately (e.g. private practitioners) or publicly-owned (e.g. zoological gardens) snake collections. In all cases, samples were from snakes that had histories, clinical signs, diagnostic test results and/or pathological findings that were consistent with a viral infection; or were from snakes that were
in-contact (either directly [e.g. cagemate] or indirectly [e.g. same room]) with the aforementioned snakes.

Three sample types were received: swabs, blood and tissue (section 4.1 – Sample Collection). Swabs were either cloacal or oral. For oral swabs, veterinarians were asked to preferentially sample the glottis and if possible, the trachea. “Blood” samples were serum, plasma or whole blood. Tissue was either fresh, freshly frozen or formalin-fixed and paraffin-embedded (FFPE).

Samples were predominantly screened for certain viruses using four different methods: haemagglutination (HA) assay, haemagglutination inhibition (HI) assay, virus isolation (VI) and polymerase chain reaction (PCR). A small selection of samples (five cloacal swabs) was also screened by a neuraminidase (NA) assay (section 5.2.2 – Neuraminidase Activity). Some samples were screened by more than one test. From 463 samples collected, 217 were screened by HA, 86 by HI, 161 by VI and 50 by PCR.

Typically, swabs were first screened with HA because it was rapid, reasonably selective for paramyxoviruses of interest (e.g. known ferlaviruses) and cost-effective to test large batches. Some swabs were additionally screened for viruses by VI and PCR. Tissue samples were usually screened by VI because it was thought that these samples would be more likely to contain intact virus. At the time, PCR was considered to be an expensive and time consuming test, especially when it was not yet known what virus, or viruses, were likely to be present. However, the sensitivity and the existence of broadly-reactive primer sets mean that PCR was an under-utilised tool at this stage of the investigation.
<table>
<thead>
<tr>
<th>States/Territories Sampled</th>
<th>Sampling Data</th>
<th>Haemagglutination Assay (HA)*</th>
<th>Haemagglutination Inhibition Assay (HI)</th>
<th>Virus Isolation (VI)</th>
<th>Polymerase Chain Reaction (PCR)#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>States/Territories</td>
<td>NSW, Qld, WA, Vic</td>
<td>NSW, Qld, WA, Vic</td>
<td>NSW, Qld, WA, SA, NT</td>
<td>NSW, Qld, WA, Vic, SA, NT</td>
</tr>
<tr>
<td></td>
<td>Collections Sampled</td>
<td>19</td>
<td>12</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Snakes Sampled</td>
<td>106</td>
<td>86</td>
<td>47</td>
<td>35</td>
</tr>
</tbody>
</table>

**Sample Type**

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Haemagglutination Assay (HA)*</th>
<th>Haemagglutination Inhibition Assay (HI)</th>
<th>Virus Isolation (VI)</th>
<th>Polymerase Chain Reaction (PCR)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloacal and Oral Swabs</td>
<td>204</td>
<td>0</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Blood</td>
<td>0</td>
<td>86</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tissue</td>
<td>13</td>
<td>0</td>
<td>149</td>
<td>23</td>
</tr>
<tr>
<td>Formalin-fixed paraffin-embedded (FFPE)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

*Table 5.1* Snake samples screened for viruses from May-2007 to October-2009. In total, 463 samples were collected from 192 snakes from 36 reptile collections from six Australian states or territories. *A second swab taken from the same snake at a different time counts as two samples from one snake. If a sample was tested by HA first and VI second, and then a sample of cell culture supernatant (from the VI attempt) was retested for HA activity, this counts as one HA sample and one VI sample. *Samples that were tested for multiple viruses by PCR, were still only recorded as one sample. NSW = New South Wales, Qld = Queensland, WA = Western Australia, Vic = Victoria, SA = South Australia, NT = Northern Territory. Samples received after PCR primers had been designed to screen for Sunshine virus by PCR (August-2010) are not included in this table. Samples were not received between October-2009 and August-2010 as efforts were focused on determining the identity of BHP1-Lung.
5.2 Screening Samples for Viruses

5.2.1 Haemagglutination Assays

From 463 samples collected for virus screening, 217 (204 cloacal or oral swabs and 13 tissue samples) were tested for the presence of a haemagglutinating agent (Table 5.1) using the methods described earlier (section 4.3 – Haemagglutination). In addition to the 217 samples, cell culture supernatant from attempts at virus isolation was sometimes tested with this haemagglutination (HA) assay. Many months after this initial HA screening, viruses were eventually isolated using virus isolation (Table 5.3) and the testing of these isolates for HA activity is discussed later (section 5.3.2 – Haemagglutination and Haemadsorbing Activity).

Out of the 217 samples that were tested for HA activity, it was detected in the swabs from two snakes that were collected from a zoological collection in Queensland in 2007 (Figure 5.1). Some snakes from this collection were occasionally displaying neurorespiratory or non-specific (e.g. lethargy) signs of disease. Sporadic deaths occurred in both symptomatic and asymptomatic animals and in these cases, histopathological assessment was consistent with a paramyxoviral infection. Furthermore, significant levels of anti-ferlaviral antibodies (haemagglutination inhibition titres up to 512/50µL) had been detected in serum samples submitted to an overseas diagnostic laboratory. With respect to the owner’s wishes, more specific details are excluded in an attempt to retain the anonymity of this collection. The first HA positive was detected in the cloacal swab of a seemingly-healthy 10 year old female black-headed python (Aspidites melanocephalus) from sampling performed in early May-2007 (sample 3) while the second positive was detected in the cloacal swab of a seemingly-healthy four year old female scrub python (Morelia amethistina) from sampling performed in late-June 2007 (sample 7).
Figure 5.1 Swabs of nine snakes (samples 1-9) from a zoological collection in Queensland. **Top:** Cloacal swabs from six snakes sampled in early May-2007. **Bottom:** Cloacal and mouth swabs from three snakes sampled in late June-2007. In both assays, an attenuated strain of human influenza virus and phosphate-buffered saline (PBS) were used as positive and negative controls respectively. Clear haemagglutination can be seen in Sample 3 and Sample 7 - Cloaca but not in any other samples.
For both of these samples (sample 3 [a cloacal sample] and sample 7 – cloaca also), the haemagglutination was still present after passing the sample through a 0.45µm syringe-tipped filter (Figure 5.2). It has been shown that some bacterial haemagglutininins are not able to be separated from the cell wall (Neter et al., 1954) and filtration should prevent haemagglutination whereas haemagglutinating viruses (e.g. paramyxoviruses) and secreted bacterial haemagglutininins would be expected to only be minimally affected by filtration. However, the significance of this result is uncertain because cell wall-associated bacterial haemagglutininins could still potentially be filterable if the cell wall had been disrupted and viral haemagglutininins may be excluded by filtration if the pore size was exclusive of the virus.

The increase in HA titre seen with Sample 7 – cloaca (32 → >256) that was observed post-filtration could be explained if the hemagglutininins in the swab sample were unevenly adsorbed to the other components of the sample (e.g. urates), potentially making each aliquot unique and therefore the inter-assay HA titres would not always be repeatable.

Figure 5.2 Swabs from two snakes (sample 3 [top] and sample 7 – cloaca [bottom], see Figure 5.1) after passing the sample through a 0.45µm filter. In both assays, an attenuated strain of human influenza virus and phosphate-buffered saline (PBS) were used as positive and negative controls respectively.
In the both cases, repeat sampling and testing of each of these two snakes at a later stage could not demonstrate the persistence of detectable haemagglutinating activity (Figure 5.3) indicating either the first result was a false positive (discussed later) or the haemagglutinating agent was no longer present in the mouth or cloaca in amounts detectable by this assay.

**Figure 5.3** Repeat sampling from two snakes (sample 3 **top** and sample 7 **bottom**, see Figure 5.1) **Top:** Mouth and cloacal swabs were collected from this snake in the morning and afternoon 56 days after the original sample was collected. Despite some unusual red cell clumping in one sample (cloaca +56d (am)), no compelling evidence of haemagglutination can be seen. **Bottom:** Mouth and cloacal swabs were taken five and seven days after the original sample was collected. In both assays, an attenuated strain of human influenza virus and phosphate-buffered saline (PBS) were used as positive and negative controls respectively.
The significance of the two positive HA titres remains undefined. A study by Khalesi et al. (2005) on *Psittacine beak and feather disease virus* (PBFDV, a parrot circovirus), concluded that six feather samples with haemagglutinating activity up to 320 HA units / 50µL of feather suspension were false-positive results because PBFDV could not be detected by polymerase chain reaction (PCR) and the HA activity could not be neutralised by anti-PBFDV antibodies and was removed by filtration through a 0.22µm filter. The HA activity of the two snake cloacal samples presented here (Figure 5.1) was not removed by filtration through a 0.45µm filter. Interestingly, neuraminidase activity could also be detected in both of these samples, consistent with a myxovirus (section 5.2.2 – Neuraminidase Activity). However, testing for anti-ferlaviral antibodies (section 5.2.3 – Haemagglutination Inhibition) and PCR testing for ferlavirus (section 5.2.6 – Polymerase Chain Reaction), and later Sunshine virus (data not shown), was negative.

### 5.2.2 Neuraminidase Activity

Only cloacal swab samples from the Queensland zoological collection discussed earlier (page 136) were selected for quantitation of neuraminidase activity. Activity was measured by assessing the amount of a neuraminidase substrate (2’-(4-methylumbelliferyl)-α-D-N-acetyleneuraminic acid) that was cleaved into a fluorescent product by the sample. Methods described earlier were followed (section 4.6 – Neuraminidase). An attenuated strain of human influenza virus (Table 4.2) and a ferlaviral isolate were used as positive controls, while cell culture medium (the medium the swabs were transported in) served as a negative control. All five cloacal swabs that were tested produced emissions at 480nm that were below those of the positive controls but above those of the negative control (Table 5.2).
Table 5.2 The neuraminidase (NA) activity present in five snake cloacal swabs collected from a zoological collection in Queensland. An attenuated strain of human influenza virus and a ferlavirus were used as positive controls while the medium that the swabs were stored in (cell culture medium) was used as a negative control. The NA substrate (2'-methyllumbrelliferyl)-α-D-N-acetylneuraminic acid) was exposed to the sample for 18 hours at 30°C. The emission at 480nm provides an estimate of the amount of NA substrate that has been cleaved into a fluorescent product. HAU = Haemagglutinating units.

<table>
<thead>
<tr>
<th>Sample (HA titre [HAU / 50µL])</th>
<th>Emission at 480nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza virus (1,024)</td>
<td>324</td>
</tr>
<tr>
<td>Ferlavirus (64)</td>
<td>268</td>
</tr>
<tr>
<td>Sample 1 (&lt; 2)</td>
<td>114</td>
</tr>
<tr>
<td>Sample 2 (&lt; 2)</td>
<td>24</td>
</tr>
<tr>
<td>Sample 3 (16)</td>
<td>177</td>
</tr>
<tr>
<td>Sample 4 (&lt; 2)</td>
<td>141</td>
</tr>
<tr>
<td>Sample 7 (32)</td>
<td>137</td>
</tr>
<tr>
<td>Cell culture medium (three replicates)</td>
<td>5.8, 5.8, 5.5</td>
</tr>
</tbody>
</table>

This experiment was adversely affected by the unsuitability of the negative control that was used. A more suitable control would have been cloacal swabs from seemingly healthy snakes. Statistical analysis may then have been needed to help demarcate significant from insignificant levels of NA activity. Steps were not taken to identify a suitable negative control and because of this, the results in this experiment cannot be validated.

It was initially thought that a limitation of this assay was that NA activity (even if filterable) could be explained by neuraminidase-secreting species of bacteria (O’Toole et al., 1971). It was therefore decided that this test was unsuitable for screening primary samples and would be reserved for samples suspected to be free of bacteria (e.g. virus isolates). In hindsight and with further consultation with the literature, it has been shown that mammalian and bacterial NA activity can be differentiated from viral NA activity through the use of ethylenediaminetetraacetic acid (EDTA) (Yolken et al., 1980). If this method was demonstrated to be applicable to snake cloacal swabs then this may have provided further insight into the significance of the results obtained here.
5.2.3 Haemagglutination Inhibition

Blood samples (plasma, serum or whole blood) were submitted from 12 Australian snake collections for quantitation of anti-ferlaviral antibodies using a haemagglutination inhibition (HI) assay described earlier (section 4.5 – Haemagglutination Inhibition). Each collection had reason to suspect the presence of a ferlavirus (e.g. clinical signs, histopathology). In total, 86 samples were tested but no titre exceeded 8 (defined as the greatest dilution that completely inhibited haemagglutination). This included serum samples that were submitted from the zoological collection in Queensland that had produced two cloacal swabs with haemagglutinating activity (page 136). Although there is disagreement within the literature as to where the specific cut-off between a positive and negative HI titre should be, titres that do not exceed 8 have always been considered negative (Jacobson et al., 1992; Jacobson and Origgi, 2007; Pasmans et al., 2007).

The results of these HI assays were marred by non-repeatable and unusual red cell clumping (Figure 4.25) and many of these assays were performed before a positive control (serum from a western diamondback rattlesnake with an HI titre of > 256 / 50µL) was obtained from Dr Rachel Marschang in Germany, and so the results presented here should be viewed cautiously. Delays in obtaining the positive control serum were caused by the time needed to obtain the necessary importation permits. When the positive control serum became available for HI testing, it performed reliably (Figure 4.21) but the unusual red cell clumping continued to occur in many samples (but never the positive control) until a new method of processing red cells was adopted (Table 4.1 and Figure 4.26). So while it may be tempting to conclude that this assay had the ability to detect a truly positive sample (the positive control) and therefore all the samples that were tested were negative, the sample results presented here should be considered equivocal until the negative predictive value of this assay is confirmed as being acceptable.
5.2.4 Electron Microscopy

Unlike haemagglutination (HA), neuraminidase (NA) and haemagglutination inhibition (HI) assays, electron microscopic examination of samples can potentially identify a wide range of viruses. Transmission electron microscopy (TEM) can evaluate the size and morphology of a virus and if examining infected cells, also the site of replication (intranuclear, intracytoplasmic or both) (Jacobson and Samuelson, 2007). More specific identification of a virus is often reliant on molecular methods such as the sequenced products of polymerase chain reaction.

The use of electron microscopy to directly examine clinical samples has been limited to only certain viruses e.g. adenoviruses in dragon cloacal washes (Jacobson and Samuelson, 2007). Identification of viral particles by electron microscopy is reliant on a number of factors: an experienced and patient pathologist (and/or electron microscopist) and an adequate quantity of virus in the specimen being examined (Jacobson and Samuelson, 2007). For these reasons, electron microscopic examination of samples that were submitted to the present study was restricted to sample types where a virus was likely to be present in high numbers.

Consideration was given to the ultrastructural examination of the surface of erythrocytes that had been haemagglutinated by cloacal swab samples (Figure 5.1) but a preliminary study using positive control material concluded that the haemagglutination titres of these samples (8-16 and 32 HA / 50µL) would be far too low to allow visualisation of viral particles (section 4.7.2 – Scanning Electron Microscopy). Furthermore, even if viral particles were found, the limited magnification and the inability to assess the internal structures of the viruses would be inadequate to identify the family that the virus belongs to. Consequently, this idea was not pursued.
The ultrastructural assessment of inclusion bodies identified by light microscopy has been used to characterise the contents of the inclusion (Jacobson et al., 1997; Jacobson and Samuelson, 2007). Samples were selected that had inclusion bodies that could be convincingly identified under light microscopy (Figures 5.4 and 5.5). Samples were processed for positive staining transmission electron microscopy (TEM) according to the methods described earlier (section 4.7.1 – Transmission Electron Microscopy).
Figure 5.4 Section of brain from a Stimson’s python (Antaresia stimsoni) displaying neurological signs. Stained with haematoxylin and eosin (H&E). **Top:** low-power view (40x). **Bottom:** Eosinophilic intranuclear inclusion bodies (arrows) can be seen in a large number of ependymal cells lining a ventricle (1,000x).
Figure 5.5 Section of brain from a south-west carpet python (Morelia spilota imbricata) displaying subtle neurological signs. Stained with haematoxylin and eosin (H&E). Top: low-power view (40x). Bottom: Massive number of eosinophilic intranuclear inclusion bodies (arrows) can be seen throughout the brain (400x).
No convincing viral particles could be detected within any inclusion body that was examined by TEM; only what appeared to be amorphous material could be seen (Figure 5.6).

**Figure 5.6** Transmission electron microscopic (TEM) view of an inclusion body in the brain of a Stimson’s python (*Antaresia stimsoni*) displaying neurological clinical signs (top). A published image of an intracytoplasmic inclusion body (IC) in a viper heart cell infected with a ferlavirus is provided for comparison (Jacobson et al., 1997) (bottom); the authors state that the inclusion body contains nucleocapsid material. Scale bar = 7µm. **Top Left:** Low power view of the nucleus of an ependymal cell showing the central inclusion body. **Right:** Higher power view of the inclusion body revealing what appears to be amorphous material.
For some viruses, the contents of its associated inclusion body can be reliably anticipated. For example, the viral inclusions associated with measles virus infection consistently contain nucleocapsid material (Ghadially, 1988). But this is not always the case as some viral inclusions are made of amorphous material (Geisbert and Jahrling, 1995). In the present study, there was no clear evidence that the inclusion bodies examined contained viral proteins (e.g. paramyxoviral nucleocapsids), but the possibility that the inclusions were still viral in nature should not be excluded. However, inclusion bodies have been seen in other reptiles that were considered to be non-viral in origin. For example, the ultrastructural appearance of the intranuclear and intracytoplasmic inclusions in the liver of a Deckert’s rat snake (*Elaphe obsoleta deckerti*) was most consistent with a type of storage material (possibly lysosomal in origin) (Jacobson et al., 1979). And in two more reports, non-viral inclusions were detected in the lung of a desert tortoise (*Gopherus agassizii*) (Jacobson and Samuelson, 2007) and the liver of a king snake (*Lampropeltis getula*) (Jacobson et al., 1980a). This means that the possibility that the inclusion bodies examined in the present study were non-viral in origin should also be considered.

Electron microscopic examination of samples is usually performed to determine the family that a virus belongs to. This information can then be utilised in molecular methods to facilitate more specific and definitive identification of the virus. Considering the equivocal results that had been obtained from electron microscopy, no further attempts to identify viral particles were made.

5.2.5 *In situ* Hybridisation

*In situ* hybridisation is a method that has allowed the detection of ferlaviral-specific nucleic acids in formalin-fixed paraffin-embedded (FFPE) samples (West et al., 2001; Sand et al., 2004). Despite numerous attempts by the author to establish a method that would reliably detect abundant housekeeping genes (e.g. 18S rRNA) in FFPE snake sections (*section 4.9 – In situ Hybridisation*), only irregular staining could be achieved inconsistently. Considering that
reliable detection of ubiquitous housekeeping genes could not be validated and moreover, that ISH requires prior knowledge of the nucleic acid sequence of the virus of interest, screening samples by ISH was not performed.

It was decided that only when a viral sequence had been determined from at least one of the snake samples being screened for viruses, would this test be revisited. When this finally occurred (approximately four years later), ISH was not performed due to time-constraints. It is intended that ISH will be used in future studies to detect Sunshine virus in FFPE material.

5.2.6 Polymerase Chain Reaction

In total, 50 samples were assessed by polymerase chain reaction (PCR) for the presence of a selection of viruses. These samples consisted of swabs (23), tissue samples (23) and formalin-fixed paraffin-embedded (FFPE) material (4). Samples were submitted from snakes with histories, clinical signs, diagnostic test results and/or pathological findings that were consistent with a virus (or viruses) that affected the neurorespiratory systems. More specifically, anti-ferlaviral antibodies (Table 3.1) and demyelinating encephalopathies with interstitial pneumonias (Figures 3.3-3.5) had been detected in some of these snakes. This meant that a paramyxovirus was of the greatest interest. However, reoviruses and retroviruses (possibly the aetiological agent of inclusion body disease (IBD) (Chang and Jacobson, 2010)) needed to be considered as well (discussed in greater detail on page 67). Due to the challenges in differentiating endogenous from exogenous retroviruses\(^5\) and the complexities in linking retroviruses to snake disease (Huder et al., 2002; Vancraeynest et al., 2006), it was decided to initially delay investigations of a retrovirus. Testing for reoviruses would become more important if a paramyxovirus could not be detected.

\(^5\) The interested reader is directed to the article by Hofacre and Fan (2010) that reviews the endogenous and exogenous forms of Jaagsiekte sheep retrovirus (JSRV).
In summary, PCR was primarily used to screen samples for ferlaviruses (genus-level detection) and more broadly, for paramyxoviruses (family-level detection). The primer sets that were used in this study were selected from published reports or were designed by the author. Each primer set would theoretically be able to detect a broad range of ferlaviruses and/or paramyxoviruses (Tables 4.3 and 4.4).

A filterable haemagglutinating agent with neuraminidase activity had been detected in two Queensland swab samples (Figures 5.1 and 5.2, Table 5.2). During the PCR testing for paramyxoviruses, particular attention was paid to these two samples. After extensive testing, none of the paramyxovirus primers designed in-house (eight single-round PCRs) nor the primers designed by Ahne et al. (1999b) (two nested PCRs) (Tables 4.3 and 4.4) yielded an amplicon of the expected size.

Of the rest of the submitted samples, only occasionally was an amplicon produced that was near to the expected size. In these situations, the PCR products were sequenced but in all cases, the Basic Local Alignment Search Tool for nucleotides (BLASTN, http://blast.ncbi.nlm.nih.gov/Blast.cgi) did not identify any similarities to the sequences in GenBank (National Center for Biotechnology Information, Bethesda, Maryland). Therefore, sequencing could not confirm that these products were of viral origin.

Samples were sometimes examined for reoviruses using the primers published by Wellehan et al. (2009). Only samples that were highly suspicious of harbouring a paramyxovirus or a reovirus (based on history, clinical signs and pathological findings), and previous testing for a paramyxovirus was negative, were tested for the presence of a reovirus. No amplicons of the expected size were produced.
5.2.7 Virus Isolation

For virus isolation, 161 samples were examined (12 cloacal/oral swabs and 149 tissue samples). Swab and tissue samples were processed and then inoculated onto susceptible cells (sometimes Vero cells but usually viper heart (VH2) cells) using the methods described earlier (section 4.2 – Virus Isolation and Propagation). Virus isolation was not attempted on the two cloacal swabs mentioned earlier (page 136). After the swabs had been used for 2-3 haemagglutination assays, a neuraminidase assay and polymerase chain reaction, insufficient sample volume remained for virus isolation. For virus isolation attempts, uninfected cells served as negative controls while two strains of ferlavirus, Fer-de-Lance virus (FDLV, ATCC VR-895) (Figure 4.3) and neotropical ophidian paramyxovirus (ATCC VR-1408) (Figure 4.4), served as positive controls. Although some curious changes to cellular morphology were seen in some virus isolation attempts (Figure 5.7), the findings were never repeatable, and after consultation with other virologists, these cells were not investigated further for the presence of viruses.

![Figure 5.7 Unusual cell morphology seen in a flask of viper heart (VH2) cells. After several days, the cells became indistinguishable from the background population. Scale bars = 100μm.](image)

Eventually, viruses were isolated from two submissions.
Sample Submission One – an Outbreak of Neurorespiratory Disease in a Private Collection from the Sunshine Coast of Queensland

In July 2008, a private breeder of birds and reptiles from Queensland acquired seven jungle carpet pythons (*Morelia spilota cheynei*) as a breeding loan to add to their collection of 70 Australian pythons (*Antaresia* sp., *Morelia spilota* ssp. and *Aspidites* sp.). Prior to the acquisition of these snakes, all the snakes in the main collection had been eating well, were in good body condition and were not displaying any overt signs of illness. Five of the seven recently-acquired snakes were emaciated, had dysecdysis (including retained spectacles) and were burdened with moderate mite infestations. For biosecurity reasons, these five snakes were humanely euthanased on the day they arrived. The remaining two snakes also had mite burdens and were treated with topical ivermectin (5mg/L). The main snake collection and the remaining two snakes (an adult male and an unsexed juvenile) that had been recently-acquired were all individually housed in a single room with the recently-acquired snakes kept separate from the main collection by several metres. Husbandry equipment was not shared between the newly-acquired snakes and the snakes in the main collection. The two groups were never attended to on the same day. A timeline of the events that unfolded in this collection are presented in Figure 5.8.

Approximately 90 days after the newly-acquired snakes arrived, while they were still being kept separate to the main collection, it was noticed that approximately one quarter of the main collection had mite infestations and were treated with ivermectin. Twenty one days later the apparently healthy, newly-acquired adult male was released into the main collection. Approximately 40 days later, there was a thermostat failure that coincided with cold weather (minima of <15°C). Seven snakes started to show neurorespiratory clinical signs consisting of a mild discharge of clear viscous fluid from the mouth, stomatitis, dyspnoea and lethargy. These snakes were treated with enrofloxacin (5mg/kg IM sid x 21 days). After an initial improvement, the snakes continued to deteriorate about 7-10 days after the completion of the course of
antibiotics. Clinical signs consisted of opisthotonos, inability to right, head tremors, independent movement of the cranial and caudal halves of the body, mild discharge of clear viscous fluid from the mouth, dyspnoea and lethargy. The most severely affected individual (\textit{M.s.cheynei} from the main collection) died a few days later. A necropsy revealed congested lung tissue but was otherwise unremarkable. Histopathology revealed demyelinating, non-suppurative encephalitis with prominent eosinophilic intranuclear inclusion bodies and rare intracytoplasmic inclusions. A section of lung revealed prominent congestion and oedema.

Several days later, the second newly-acquired snake (\textit{M.s.cheynei}, while still in quarantine) developed acute neurorespiratory clinical signs and died two days later. Gross pathology and histopathological findings were similar to the first death. Approximately seven days later, another seven snakes started to display similar neurorespiratory clinical signs. The previously affected snakes were continuing to display mild chronic neurorespiratory clinical signs. Approximately another seven days later, the decision was made to depopulate, so the 70 remaining snakes were humanely euthanased.

Samples were retrieved from 17 snakes (selection was based on clinical signs and/or which snakes they had been in direct contact with). The brain and a portion of lung, liver and kidney were collected from 16 of these snakes. Half of the organ sample was frozen immediately and the other half was placed into 4\% (v/v) formalin. The brain was not submitted from the seventeenth snake but the rest of the organ samples were collected identically. Blood was collected from 13 of these 17 snakes, and serum was then frozen down. Frozen organ samples and serum were submitted (on dry ice, using an overnight courier) to the author for further testing. After 24 hours of fixation, formalin was decanted from the “wet” formalin-fixed organ samples and the samples were submitted to the author using regular postage.
Figure 5.8 Timeline of events of a private collection of pythons from the Sunshine Coast in Queensland starting from the acquisition of seven jungle carpet pythons (*Morelia spilota cheynei*) and finishing when the entire collection was humanely euthanased several months later.
Sample Submission Two – Samples collected by Dr Cathy Shilton from the Berrimah Veterinary Laboratories (BVL) in Darwin, Northern Territory

From 2005 to 2008, Dr Cathy Shilton, an anatomical veterinary pathologist from the Northern Territory (NT), identified nine snakes from four separate NT locations that had histories, clinical signs and/or pathological findings that were considered consistent with a viral (potentially paramyxoviral) infection. Formalin-fixed paraffin-embedded (FFPE) material was available from all nine snakes. Additionally, samples from three brains and two lungs were collected from four snakes, representing one of these sources. These samples were stored at -80°C before being submitted on dry ice to the author.

Cytopathic Effects

In total, virus isolation was attempted on 161 samples (12 swabs and 149 tissue samples), 76 of which were from Sample Submissions One and Two (67 and nine samples respectively). Samples were processed and then inoculated onto viper heart (VH2) cells as previously described (section 4.2 – Virus Isolation and Propagation). The kidney and liver samples from each snake from Sample Submission One (Sunshine Coast, Queensland) were pooled to save time and reagents. After the clarified tissue suspensions from clinically-affected snakes had been inoculated onto VH2 cells, the cells were examined daily for cytopathic effects (CPE). Of the 161 samples that were examined by virus isolation, CPE was detected in nine samples from five snakes (Table 5.3). All five snakes were from Sample Submissions One or Two. In all cases, CPE was characterised by extensive formation of multinucleate giant cells (Figures 5.9 and 5.10). Very little cell lysis was noted irrespective of the method of blind passage (freeze-thaw or trypsinisation) or the length of time (up to three weeks) that the cells were left to incubate after the emergence of CPE (data not shown). During these experiments, cell rounding, cell detachment and perinuclear granulation were not significant hallmarks of this virus’s CPE. Cytoplasmic vacuolation was seen occasionally (Figure 5.10).
<table>
<thead>
<tr>
<th>Species: Abbreviation</th>
<th>Sample Origin</th>
<th>Clinical History</th>
<th>Virus Isolation (first appearance of CPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Jungle carpet python (Morelia spilota cheynei): JCP1Qld</td>
<td>Sunshine Coast, Queensland</td>
<td>Subtle neurological signs. Shared enclosure with snake that died acutely</td>
<td>+ (p2d2)</td>
</tr>
<tr>
<td>Black-headed python (Aspidites melanocephalus): BHP1</td>
<td>Sunshine Coast, Queensland</td>
<td>Low grade respiratory disease and stomatitis. Shared enclosure with two snakes (one after the other) that died acutely</td>
<td>+ (p2d2)</td>
</tr>
<tr>
<td>Jungle carpet python 1: JCP1NT</td>
<td>Northern Territory</td>
<td>Several snakes from this collection showed dysecdysis, anorexia, neurological signs and/or death over several weeks. This snake was euthanased while still relatively active</td>
<td>+ (p1d5)</td>
</tr>
<tr>
<td>Jungle carpet python 2: JCP2NT</td>
<td>Northern Territory</td>
<td>Died naturally</td>
<td>-</td>
</tr>
<tr>
<td>Jungle carpet python 3: JCP3NT</td>
<td>Northern Territory</td>
<td>Moribund</td>
<td>+ (p1d6)</td>
</tr>
</tbody>
</table>

Table 5.3 Positive virus isolation results from snakes from a source of samples submitted from the Sunshine Coast, Queensland and Berrimah Veterinary Laboratories, Northern Territory. Positive virus isolation was defined by characteristic cytopathic effects (CPE) (large syncytium with minimal cell lysis). - = negative. + = positive. n.s. = not sampled. pXdY = the passage number and days since the last passage. The day of cell inoculation = p1d1.
Figure 5.9 Uninfected viper heart (VH2) cells (above) and VH2 cells infected with the supernatant of a lung suspension from a black-headed python (Aspidites melanocephalus) that died with neurorespiratory signs of disease (bottom). Extensive formation of multinucleate giant cells can be seen in the infected culture. Scale bars = 100µm.
Figure 5.10 Viper heart (VH2) cells infected with the supernatant of a brain suspension from a jungle carpet python (*Morelia spilota cheynei*) that died with neurorespiratory signs of disease. Extensive formation of multinucleate giant cells and a cell with a large amount of intracytoplasmic vacuolation (arrow) can be seen. Scale bar = 100µm.

Supernatant from viper heart (VH2) cells that were infected with a lung suspension from a black-headed python from the Sunshine Coast (BHP1-Lung, Table 5.3) was selected for additional testing in an effort to further characterise this isolate. This isolate was selected because it produced widespread CPE and was from a snake that was positive by virus isolation in all organ samples tested. The selection of the isolate from the lung, as opposed to the brain or kidney-liver, was arbitrary.

Three passages of BHP1-Lung (p1 → p4) in viper heart cells demonstrated that the CPE could be observed repeatedly (Figure 5.11). The viral titre of BHP1-Lung (at the fourth passage) was calculated using the Reed-Muench method as previously described (section 4.2 – Virus Isolation and Propagation). The tissue culture infectious dose (TCID\(_{50}\)) was calculated as \(10^{2.75}\).
mL⁻¹. This virus was sent to the veterinary virology laboratory at the Berrimah Veterinary Laboratories (BVL) in the Northern Territory for archiving and continuing investigation. In the hands of one of the virologists at this laboratory, a viral titre (TCID₅₀), using the same VH2 cell line, of at least (further serial dilutions not performed) 10⁵ mL⁻¹ was obtained (Richard Weir, pers. commun.). So it is possible that this virus does not inherently replicate at a low viral titre but instead may be sensitive to its growth conditions.
Figure 5.11 Uninfected viper heart (VH2) cells (top image) compared to BHP1-Lung throughout three passages (bottom two rows). The third day of the second passage (p2d3) shows early cytopathic effects (CPE) in the form of syncytial cell formation. Three days later (p2d6), further progression of CPE can be seen. Syncytial cells are larger and there is considerable cytoplasmic clearing around a central cluster of nuclei. Similar CPE can be seen during the next two passages (p3d7 and p4d8). Scale bars = 100µm.
Once the CPE could be reliably anticipated, a short experiment was set up to compare two different methods of viral passage: trypsinisation and freeze-thawing. For trypsinisation, the virus was passaged by trypsinising and splitting infected cells (one to three split). For the freeze-thaw method, the virus was passaged by freezing, thawing, clarifying and then inoculating infected cell lysate onto uninfected cells. For each of these two methods, the viral passage was performed at two different intervals. For the short interval, once CPE could be seen in 10% of the monolayer (four days), the virus was passaged, and then again two days later. For the long interval, the virus was passaged two days later than the short interval (six days).

Passaging the virus using the trypsinisation method appeared to outperform the freeze-thaw method as it clearly augmented the percentage of the monolayer that was displaying CPE (Table 5.4 and Figure 5.12). However, this experiment did not involve several passages, was not repeated, did not use any replicates for each passage method and only crudely assessed viral titre by using CPE as a surrogate marker. So although there appears to be a clear advantage by passaging the virus by splitting the infected cells, the results of this short experiment should not be over-interpreted. This experiment was not able to provide an optimal passage interval or an optimal splitting ratio (e.g. 1:2, 1:3, 1:4 etc).

It has been shown that Fer-de-Lance virus contains a fusion gene that is expressed as a precursor protein ($F_0$) which is then cleaved by the ubiquitous host cell enzyme, furin, into active subunits (Franke et al., 2006). In 1996, Richter et al. described the use of trypsin to improve the in vitro infectivity of three strains of ferlavirus. It was presumed that the improved infectivity was caused by trypsin cleaving the inactive fusion protein precursor into its active subunits. The trypsinisation method seemed to improve the infectivity of BHP1-Lung and this may have been attributable to the presence of trypsin.
<table>
<thead>
<tr>
<th>Passage method</th>
<th>Days since start of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Freeze-thaw: short interval</td>
<td>0%</td>
</tr>
<tr>
<td>Freeze-thaw: long interval</td>
<td>0%</td>
</tr>
<tr>
<td>Trypsinisation: short interval</td>
<td>0%</td>
</tr>
<tr>
<td>Trypsinisation: long interval</td>
<td>0%</td>
</tr>
</tbody>
</table>

**Table 5.4** Percentages of viper heart (VH2) cell monolayers displaying cytopathic effect following inoculation of BHP1-Lung. Four methods of viral passage are compared. **Viral passages are underlined and displayed in bold type** and for these days, the percentage is representative of the CPE of the monolayer before the passage.
Figure 5.12 Viper heart (VH2) cells infected with BHP1-Lung. Above: Two days after BHP1-Lung-infected VH2 cells were split 1:3 (10% of monolayer was showing cytopathic effects [CPE] before this split). Approximately 50% of the monolayer is displaying CPE in the form of syncytial cell formation (arrows). Below: Two days after BHP1-Lung-infected cells (CPE was also 10%) were frozen, thawed, clarified and then the supernatant was inoculated onto uninfected VH2 cells. Only about 1% of the monolayer is displaying subtle signs of CPE. A small multinucleate cell can be seen (arrow). Scale bars = 100µm.
A brief attempt was made to assess the infectivity of BHP1-Lung in a second cell line. BHP1-Lung was inoculated onto Vero cells using the methods described earlier for a freeze-thaw passage. Briefly, viper heart (VH2) cells infected with BHP1-Lung were frozen, thawed, clarified and then the supernatant was inoculated onto Vero cells. Inoculated cells were assessed daily for cytopathic effects (CPE) and after two weeks, a freeze-thaw passage was repeated. CPE could not be detected in Vero cells. Many paramyxoviruses are known to have a broad *in vitro* host range (Lamb *et al.*, 2005) and ferlaviruses, for example, are capable of replication in Vero cells (Mayr *et al.*, 2000) (*Figure 4.4*). This experiment was performed before it was shown that BHP1-Lung seemed to produce more widespread CPE when passaged using the trypsinisation method. Future experiments that aim to define the *in vitro* host range of BHP1-Lung should consider using a method that includes exposure to trypsin. This short study was not pursued as physicochemical investigations that might have been able to provide insight into the viral family that BHP1-Lung belongs to were seen as higher priorities.

After attempting virus isolation from 161 samples, a virus (or viruses) had been isolated into viper heart (VH2) cells that repeatedly produced cytopathic effects (CPE) in the form of widespread syncytial cell formation with minimal cell lysis. Based on the CPE seen, it was considered most likely that BHP1-Lung would be a member of one of four families of syncytial cell-forming viruses: reovirus, paramyxovirus, retrovirus or herpesvirus. Reovirus and paramyxovirus infections and inclusion body disease (potentially caused by a retrovirus (Schumacher *et al.*, 1994)) have all been implicated in neurorespiratory disease in snakes (Marschang and Chitty, 2004; Ritchie, 2006; Jacobson, 2007). Although a herpes-like virus was isolated from a green iguana (*Iguana iguana*) that caused syncytial cell formation in cell culture (Clark and Karzon, 1972), the histories, clinical signs and pathological findings of the five snakes (*Table 5.3*) that a virus (including BHP1-Lung) was isolated from, were inconsistent with a herpesvirus infection.
5.2.8 Summary of Sample Testing

To this point, testing 217 samples by HA, 5 by NA, 86 by HI and 50 by PCR had produced only two interesting results: two cloacal swabs that each contained a filterable haemagglutinating agent and also possessed NA activity. Examination of inclusion bodies by TEM had also failed to identify a virus. Screening 161 samples by VI had produced (by far) the most compelling results: nine viruses were isolated that all produced similar CPE. One of these isolates was selected for further testing, BHP1-Lung, and this additional testing is discussed in the remainder of this chapter.

5.3 Laboratory Investigations of BHP1-Lung

5.3.1 Electron Microscopic Examination

Examination of the ultrastructure of BHP1-Lung could potentially allow identification of the viral family it belongs to. BHP1-Lung had repeatedly shown the ability to cause syncytial cell formation and this suggested that it was most likely to be a reovirus, paramyxovirus, retrovirus or herpesvirus. These four viruses all have characteristic ultrastructural appearances (Table 5.5). Images can be reviewed elsewhere: for ferlaviruses see Figure 2.8; for reptilian orthoreoviruses and a mammalian orthoreovirus (Nelson Bay orthoreovirus, NBV), see Ahne et al. (1987b) and Figure 4.30 respectively; for retroviruses, see Linial et al. (2005); and for herpesviruses, see Davison et al. (2005).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Orthoreovirus</th>
<th>Ferlavirus</th>
<th>Retrovirus</th>
<th>Herpesvirus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td>icosahedral (but can appear spherical), spiked at the vertices, “cygnet ring” appearance, 60-80nm</td>
<td>pleomorphic, spiked, “herring bone” appearance to nucleocapsid protein, 30-500nm</td>
<td>spherical, small surface projections, 80-100nm</td>
<td>characteristic appearance, spherical, ~200nm</td>
</tr>
<tr>
<td><strong>Buoyant density</strong></td>
<td>1.36g/cm(^3) in CsCl</td>
<td>1.13-1.18g/cm(^3) in sucrose</td>
<td>1.16-1.18g/cm(^3) in sucrose</td>
<td>1.22-1.28g/cm(^3) in CsCl</td>
</tr>
</tbody>
</table>

Table 5.5 Descriptions of the ultrastructural appearances of orthoreoviruses (Chappell et al., 2005), ferlaviruses (Richter et al., 1996), retroviruses (Linial et al., 2005) and herpesviruses (Davison et al., 2005).

Being able to visualise reptilian viruses by transmitting electron microscopy (TEM) requires a skilled and patient pathologist and the likelihood of success will be improved if the virus is abundant (Jacobson and Samuelson, 2007). Prior to visualisation by electron microscopy, viruses can be concentrated by ultracentrifugation. Additionally, the virus can also be purified by ultracentrifuging the virus in a density gradient of either caesium chloride (for isopycnic centrifugation) or sucrose (for rate-zonal centrifugation) (Mahy and Kangro, 1996). Viruses will equilibrate into specific areas along this gradient and this point can be used to help determine the viral identity (Table 5.5).

Several different sample types of BHP1-Lung were examined by TEM. Unprocessed, clarified, clarified/PEG-precipitated and clarified/ultracentrifuged culture media, and clarified frozen-thawed cell lysate were all examined by TEM after negative staining with phosphotungstic acid. TEM was also used to examine intact infected cells after positive staining with uranyl acetate and lead citrate. Using the trypsinisation method (page 161 and Figure 5.12), cells exhibiting extensive cytopathic effects (CPE) could be examined. The methods for sample processing and staining were described earlier (section 4.7 – Electron Microscopy).

For all samples of BHP1-Lung that were examined by positive or negative staining TEM, no viral particles could be confidently identified after extensive searches. The most interesting image
of a “virus-like” particle is presented in Figure 5.13. Unprocessed cell culture supernatant from
a flask infected with JCP$_{NT}$-Brain (jungle carpet python 1 (Northern Territory) – brain) (Table
5.3) was also extensively examined and no viral particles could be detected.

![Figure 5.13](image)

Electron microscopy generally requires a high viral titre. The low viral titre achieved in this
study (Tissue Culture Infectious Dose (TCID$_{50}$) = $10^{2.75}$ mL$^{-1}$) may help explain why viral particles
could not be found. In anticipation of this concern, samples of BHP1-Lung were concentrated
using several methods and the supernatant of a separate sample (JCP$_{NT}$-Brain) of infected
viper heart cells were examined but viral particles continued to elude detection.
As mentioned earlier, identification of viral particles by electron microscopy is reliant on a number of factors: an experienced and patient pathologist (and/or electron microscopist) and an adequate quantity of virus in the specimen being examined (Jacobson and Samuelson, 2007). To this end, the failure to identify any viral particles in samples infected with BHP1-Lung may have been attributable to the relative inexperience of the author in electron microscopy. Outsourcing positively-staining grids of infected cell culture and negatively-stained grids of cell culture supernatant to more experienced electron microscopists (e.g. Dr Alex Hyatt at the Australian Animal Health Laboratories [AAHL]) may have been helpful.

5.3.2 Haemagglutination and Haemadsorbing Activity

BHP1-Lung had repeatedly shown the ability to cause syncytial cell formation in cell culture and this suggested that it was most likely to be a reovirus, paramyxovirus, retrovirus or herpesvirus.

Ferlaviruses (all previously discovered reptilian paramyxoviruses) have the capability to haemagglutinate a selection of vertebrate erythrocytes (Richter et al., 1996; Kolesnikovas et al., 2006), however, many paramyxoviruses outside of the proposed ferlavirus genus have not shown any haemagglutinating activity (Lamb et al., 2005). All reoviruses that have been detected in reptiles have been tentatively assigned to the genus Orthoreovirus (Marschang, 2011) and although some mammalian orthoreoviruses have demonstrated haemagglutination capabilities (e.g. Mammalian orthoreovirus 1) (Chappell et al., 2005), no reptilian orthoreovirus has shown this trait (Ahne et al., 1987b; Vieler et al., 1994; Lamirande et al., 1999; Chappell et al., 2005). Retroviruses have not been shown to have any haemagglutinating activity. Feline and canine herpesviruses have been shown to possess haemagglutination activity (Gillespie and Judkins, 1974; Xuan et al., 1990) but the author was unable to find any investigations trying to identify the ability of reptilian herpesviruses to haemagglutinate erythrocytes. In summary, if BHP1-Lung was found to have haemagglutinating activity, it would raise the
suspicion that BHP1-Lung was a paramyxovirus (likely ferlavirus) and reduce the likelihood that it was a reovirus, retrovirus or herpesvirus.

The haemagglutinating ability of BHP1-Lung was investigated by (1) assessing the *in vitro* macroscopic haemagglutination (HA) of guinea pig and chicken erythrocytes under various conditions and (2) assessing the haemadsorbing (Hd) traits of viper heart cells infected with BHP1-Lung.

Previously, samples were screened for the presence of a haemagglutinating agent using only chicken erythrocytes but to broaden the scope of this assay, the choice of erythrocyte was reviewed. For ferlaviruses at least, the reliability of haemagglutination has been dependent on what erythrocytes were used (Richter *et al*., 1996). The decision was made to use guinea pig and chicken erythrocytes because these are the two species that have been used in HA and haemagglutination inhibition (HI) assays for ferlaviruses both in diagnostic laboratories and in the published literature. Guinea pig erythrocytes are used by the Texas Veterinary Medical Diagnostic Laboratory and have also been used by various authors (Clark *et al*., 1979; Richter *et al*., 1996; Allender *et al*., 2006). Chicken erythrocytes are used by the University of Florida in the USA and Honhenheim University in Germany. They were also selected by several authors of published reports (Clark *et al*., 1979; Jacobson *et al*., 1980b; Jacobson *et al*., 1981; Potgieter *et al*., 1987; Jacobson *et al*., 1991; Jacobson *et al*., 1992; Blahak, 1995; Richter *et al*., 1996; Jacobson *et al*., 1997; Gravendyck *et al*., 1998; Franke *et al*., 2001; Jacobson *et al*., 2001a).

Three samples from a flask of viper heart (VH2) cells infected with BHP1-Lung were assessed for haemagglutination (HA) activity. These assays were performed in accordance with the methods described previously (**section 4.3 - Haemagglutination**). Untreated supernatant formed the first sample and HA activity was assessed using chicken erythrocytes (**Figure 5.14**). The next two samples were taken from clarified frozen-thawed cell lysate, one of which was
additionally concentrated by polyethylene glycol (PEG) precipitation (Figure 5.15). These three samples were then added to chicken and/or guinea pig erythrocytes and assessed for HA activity. Untreated ferlavirus, and ferlavirus that had been concentrated using PEG-precipitation (Figure 4.17), served as positive controls for this assay. Phosphate-buffered saline (PBS) was used as a negative control.

Other samples were also analysed. In addition to BHP1-Lung, the unprocessed supernatants of VH2 cells that were infected with samples from Queensland and the Northern Territory (Table 5.3) were also assessed for HA activity (Figures 5.14 and 5.16).

**Figure 5.14** Haemagglutination activity of the supernatant from four flasks of viper heart (VH2) cells with syncytial cell formation. Isolates were at the fourth day of their second passage. PBS = Phosphate-buffered saline. Abbreviations for each sample are defined in Table 5.3.

**Figure 5.15** Haemagglutination (HA) activity of BHP1-Lung after concentration using polyethylene glycol (PEG)-precipitation. Both guinea pig and chicken erythrocytes (RBCs) were used as indicators of HA activity.
activity. This experiment was performed before a new erythrocyte processing method (Figure 4.26) was being used routinely and so although the first two wells of BHP1-Lung using the guinea pig erythrocytes appear to represent clumped red cells (Figure 4.25), they could be displaying haemagglutination.

Figure 5.16 Haemagglutination activity (using guinea pig erythrocytes) of the supernatant from eight flasks of viper heart (VH2) cells with syncytial cell formation. Supernatant was collected at the end of the first (p1) and second (p2) passage. PBS = Phosphate-buffered saline. Abbreviations for each sample are defined in Table 5.3.

HA activity of BHP1-Lung could not be detected by these assays. If present, it was below the lower limit of detection of this assay (<2 HA units / 50µL) and even after concentration with PEG-precipitation, HA activity could not be confidently detected. Other concentration methods could have been used (e.g. ultrafiltration, ultracentrifugation), and for each method, different vertebrate erythrocytes and different incubation conditions (e.g. room temperature or 4°C) could have been trialled, but the experiments presented here do not provide compelling evidence that BHP1-Lung contains any HA activity.

For haemadsorption (Hd), a flask of viper heart cells infected with BHP1-Lung was assessed using the methods described earlier (section 4.4 – Haemadsorption). A flask of Vero cells
infected with ferlavirus was used a positive control and a flask of uninfected viper heart cells was used as a negative control. Only minimal erythrocyte attachment was seen with the Vero cells that were infected with ferlavirus and no erythrocytes could be seen that were attached to the monolayer of BHP1-Lung-infected or uninfected viper heart cells.

Overall, these experiments were unable to demonstrate that BHP1-Lung contained any haemagglutinating (either HA or Hd) activity. It may have been helpful to determine the lowest infectious dose (TCID₅₀) that could be detected using this HA assay. This may have shown that the concentration of BHP1-Lung (previously determined by its infectious dose (TCID₅₀) of 10⁻³.⁷⁵ mL⁻¹) was below the lower limit of detection for the HA assay and as a result the assay would be unable to detect the HA activity of BHP1-Lung even if it was present. Determining a tissue culture infectious dose utilises virus replication and as such its sensitivity can be superior to methods such as haemagglutination assays where a very large number of particles are needed to produce the observable effect (Mahy and Kangro, 1996). Clearly, the lower limit of detection of the HA assay (expressed as a TCID₅₀) cannot be determined for BHP1-Lung (because HA activity could not be detected) and so a virus with haemagglutinating activity that could be reliably detected would need to be used (e.g. ferlavirus). However, the correlation between HA activity and TCID₅₀ for a particular ferlavirus may not be the same for BHP1-Lung (if it had HA activity), so care would be needed when extrapolating from the chosen ferlavirus to BHP1-Lung. So in summary, it could not be shown that BHP1-Lung contained any HA or Hd activity but this should not be interpreted to mean that it was absent. Without phenotypic evidence for the haemagglutinating activity of BHP1-Lung, molecular identification of this trait would be needed (section 6.2 – Partial Genome Characterisation).

Had it been shown that BHP1-Lung was a haemagglutinating virus; neuraminidase activity could have been tested by assessing the haemagglutination of erythrocytes that had been previously exposed to BHP1-Lung. If BHP1-Lung contained a receptor destroying enzyme (neuraminidase), erythrocytes would not be able to be haemagglutinated for a second time.
(Clark et al., 1979). But because HA activity could not be detected in BHP1-Lung, this experiment could not be attempted.

5.3.3 Neuraminidase Activity

As mentioned previously, BHP1-Lung had repeatedly shown the ability to cause syncytial cell formation in cell culture and this suggested that it was most likely to be a reovirus, paramyxovirus, retrovirus or herpesvirus. Amongst viruses, neuraminidase activity is unique to orthomyxoviruses and paramyxoviruses (Kawaoka et al., 2005; Lamb et al., 2005). Although neuraminidase activity has not been detected in paramyxoviruses from the subfamily Pneumovirinae (e.g. Avian metapneumovirus) or the genera Morbillivirus (e.g. Rinderpest virus, Canine distemper virus) and Henipavirus (e.g. Hendra virus, Nipah virus) (Lamb et al., 2005), all ferlaviral isolates tested so far have demonstrated neuraminidase activity (Marschang, 2011). The presence of neuraminidase activity in BHP1-Lung would suggest that it was a paramyxovirus (likely ferlavirus) and not a reovirus, retrovirus or herpesvirus.

The neuraminidase activity of BHP1-Lung was investigated in two experiments by assessing the amount of a neuraminidase substrate, \(2'-(4\text{-methylumbelliferyl})-\alpha\text{-D-N-acetylneuraminic acid}\), that was cleaved into a fluorescent product by the supernatant of infected viper heart cells. To do this, methods described earlier were followed (section 4.6 – Neuraminidase). A ferlaviral isolate were used as positive controls, while uninfected culture substrate served as a negative control. In the first experiment, BHP1-Lung and ferlavirus were left to incubate with the neuraminidase substrate for either 30 minutes or one hour (Table 5.6). The second experiment was the same as the first except that the second incubation time was doubled to two hours (Table 5.6). Previously it had been shown that a longer incubation time produced a greater emission at 480nm (Table 4.2).

6 Like all members of the genus Morbillivirus, the attachment protein of Rinderpest virus is classified as an H protein (as compared to an HN protein) as it is not considered to have significant neuraminidase activity (Lamb et al., 2005). However, in the presence of a highly specific substrate, bovine submaxillary mucin, neuraminidase activity has been detected (Langedijk et al., 1997).
#### Table 5.6

<table>
<thead>
<tr>
<th>Sample</th>
<th>Emission at 480nm [first replicate, second replicate (average reading)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment One</strong></td>
<td></td>
</tr>
<tr>
<td>Incubation time</td>
<td>30mins</td>
</tr>
<tr>
<td>Ferlavirus</td>
<td>297, 314 (305.5)</td>
</tr>
<tr>
<td>Uninfected allantois</td>
<td>0, 0 (0)</td>
</tr>
<tr>
<td>BHP1-Lung</td>
<td>22, 25 (23.5)</td>
</tr>
<tr>
<td>Uninfected cell culture supernatant</td>
<td>3, 9 (6)</td>
</tr>
</tbody>
</table>

| **Experiment Two**                  |                                                                        |
| Incubation time                     | 30mins | 2hrs                              |
| Ferlavirus                          | 300, 320 (310) | 297, 313 (305)                    |
| Uninfected allantois                | 0, -3 (-1.5) | 0, -3 (-1.5)                      |
| BHP1-Lung                           | 17, 21 (19) | 16, 20 (18)                       |
| Uninfected cell culture supernatant | 0, 6 (3) | -1, 5 (2)                         |

These two experiments demonstrated that under these conditions the neuraminidase activity of BHP1-Lung was less than a ferlaviral isolate and that the amount of neuraminidase product did not increase after a two hour incubation period.

To investigate the possibility that the relatively (compared to a ferlaviral isolate) low level of detectable neuraminidase activity (if any at all) might be due to the low titre of BHP1-Lung (TCID$_{50}$ = $10^{2.75}$ mL$^{-1}$), attempts were made to determine the lower limit of detection of this assay. To do this, a ferlaviral isolate was serially diluted and maximum incubation periods were extended from two hours to 24 hours (Figure 5.17). For the ferlaviral isolate, there was an almost-geometric decrease in the emission at 480nm as the dilution increased from undiluted to a 15,625 dilution. And this was observed for each time point: 50m, 2h20m, 3h40m and 20h. Additionally, starting at the 25-fold dilution and continuing to the 15,625-fold dilution, longer incubation periods were associated with higher 480nm emissions. In brief, Figure 5.17 shows
that ferlaviral emission signals were positively correlated with incubation time but negatively correlated with isolate dilution.

In contrast to these findings, the emission signals from all concentrations of BHP1-Lung actually slightly decreased with increasing incubation times. This finding is not consistent with neuraminidase activity in BHP1-Lung. The ferlaviral isolate had an initial dilution of 128 haemagglutinating units per 50 µL (128HA/50µL) and so if neuraminidase activity was truly detected at the greatest dilution, 1:15,625, than this would suggest that a ferlaviral isolate with an HA titre of less than 0.008HA/50µL can be detected by this assay. This would make the lower limit of detection at least 250-times lower than a haemagglutination assay (2HA/50µL divided by 0.008HA/50µL). However, these methods have not been able to detect influenza virus below an infectious dose (TCID$_{50}$) of $10^3$ mL$^{-1}$ (Yolken et al., 1980), which is still greater than the TCID$_{50}$ of BHP1-Lung ($10^{2.75}$ mL$^{-1}$). So it remains unclear whether the concentration of BHP1-Lung exceeded the lower limit of detection of this assay and therefore, the neuraminidase activity inherent to BHP1-Lung remains uncertain.
Figure 5.17 The amount of neuraminidase (NA) activity of five-fold serial dilutions of BHP1-Lung compared to a strain of ferlavirus (ATCC VR-1408, 128HA / 50µL). The NA substrate (2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid) is exposed to each isolate for a certain length of time (50 minutes [50m], two hours and 20 minutes [2h20m], three hours and 40 minutes [3h40m] or 20 hours and 20 minutes [20h]) to produce a fluorescent substrate. The emission at 480nm provides an estimate of each isolate’s NA activity.
A solid-phase neuraminidase assay with a sensitivity that provides a 100-fold improvement on the methods utilised here has been described (Yolken et al., 1980). It is a modified enzyme-linked immunosorbent assay (ELISA) and was not used in this project due to the difficulty and expense in obtaining the necessary reagents. Another neuraminidase assay that utilises a 1,2-dioxetane derivative of sialic acid (neuraminic acid) has been described that provides a 67-fold reduction in the lower limit of detection of NA activity when compared against methods that utilise the neuraminidase substrate used in this project (Buxton et al., 2000). Although these methods have not been validated for the detection of ferlaviral neuraminidase, future studies should consider them.

As mentioned earlier, the attachment protein of paramyxoviruses from the genus *MORBillivirus* (H) has been thought to only possess haemagglutinating activity (Lamb et al., 2005). However, neuraminidase activity has been demonstrated in *Rinderpest virus* (a morbillivirus) but only when exposed to bovine submaxillary mucin (Langedijk et al., 1997). The implication of this finding is that even if sensitive methods of detecting neuraminidase activity were applied to higher titres of BHP1-Lung in the future, a result that does not demonstrate the presence of neuraminidase activity should be cautiously interpreted.

This short phenotypic study had failed to unequivocally detect neuraminidase activity in BHP1-Lung and so the presence of this enzyme would be reliant on molecular identification.

5.3.4 Haemagglutination Inhibition

BHP1-Lung was collected from a privately-owned snake collection in Queensland during an outbreak of neurorespiratory disease (page 152). Serum was collected from 13 of these snakes and was available for testing for anti-ferlaviral antibodies using a haemagglutination inhibition (HI) assay. The HI assay was performed in accordance with the methods previously described (section 4.5 – Haemagglutination Inhibition). Many samples contained haemolysis (Figure 4.18) and clots (fibrin and whole blood, Figure 4.19). Consequently, these samples required
extra processing that was described earlier (section 4.5 – Haemagglutination Inhibition). It was previously shown that the affect on HI titre that these changes had was increasing the difficulty in assessing the settling of erythrocytes in “serum” samples containing large amounts of haemoglobin (Figure 4.27).

Using a neotropical strain of ophidian paramyxovirus (ATCC VR-1408) as antigen, no HI titre exceeded 8 (Figure 5.18).

*Figure 5.18* Haemagglutination inhibition (HI) assay of serum samples collected from a privately-owned collection of snakes that produced the isolate BHP1-Lung (sample 7 in this figure). The inadequate separation of erythrocytes from serum prior to freezing has provided varying shades of red in some wells. No HI titre exceeded eight. HA = haemagglutination.
In 2008, Allender et al. used serum samples from 26 eastern massasaugas (*Sistrurus catenatus catenatus*, a North American pit viper) to demonstrate the considerable variability in ferlaviral HI antibody testing that existed between three American diagnostic laboratories. Many variables contribute to inter-laboratory variation in HI titre (e.g. the strain of virus and the number of haemagglutinating units of antigen). A more detailed discussion of the variables that influence HI titre in reptiles was described earlier on page 52. The serum samples in the present study were tested against an American isolate of ferlavirus (ATCC VR-1408) but it has been shown that not all ferlaviruses are serologically related to each other. It has been suggested that at least some European isolates may be serologically unrelated to some American isolates (Lloyd et al., 2005). It was for this reason that these serum samples were tested against the second isolate of ferlavirus available to this study (Fer-de-Lance virus, ATCC VR-895, a European isolate). Results of this second assay were uninterpretable because of abnormal red cell clumping (similar in appearance to Figure 4.25). The assay was not repeated.

Various titres have been used to demarcate previous ferlaviral exposure from no previous exposure (Lloyd and Flanagan, 1991; Gravendyck et al., 1998) but the titres in this report (≤8) are generally considered to be non-specific. The present study is limited by the lack of paired serum samples (where the second sample is collected at an interval that exceeds the period of convalescence). Theoretically, the single sample point tested in this study may have been taken before a detectable antibody response could be measured. A second sample may have been able to demonstrate a rising titre suggesting active infection. So although this testing could not demonstrate the presence of anti-ferlaviral antibodies, a number of confounding variables mean the results presented here should not be over interpreted.

### 5.3.5 Chemical Experiments

Chemical experimentation of BHP1-Lung would assess the changes to viral titre after the isolate was exposed to chloroform and ether; a DNA synthesis inhibitor; and an acidic environment.
BHP1-Lung had repeatedly shown the ability to cause syncytial cell formation in cell culture and this suggested that it was most likely to be a reovirus, paramyxovirus, retrovirus or herpesvirus. These four viruses all have a unique panel of chemical traits. These include, but are not limited to, the ability to haemagglutinate vertebrate erythrocytes, neuraminidase activity, the presence or absence of a lipid envelope and an RNA or DNA genome. At the same time as an investigation of the chemical traits of BHP1-Lung, several polymerase chain reaction (PCR) primer sets would be tested against this isolate (section 5.2.6 – Polymerase Chain Reaction). Although a sequenced PCR product would be more likely to specifically identify the viral family that BHP1-Lung belongs to, investigation of its chemical traits does not require any prior knowledge. A summary of the chemical traits of the orthoreoviruses (all known reptilian reoviruses), ferlaviruses (all known reptilian paramyxoviruses), retroviruses and herpesviruses is presented in Table 5.7. This is followed by a discussion of each characteristic.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Orthoreovirus</th>
<th>Ferlavirus</th>
<th>Retrovirus</th>
<th>Herpesvirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid envelope</td>
<td><strong>No</strong> but can bud from a cell and transiently have an envelope</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Haemagglutination</td>
<td>Species-dependent but absent in reptilian orthoreoviruses</td>
<td><strong>Yes</strong> but genus-dependent for other paramyxoviruses</td>
<td>No</td>
<td>Species-dependent but not reported for reptilian herpesviruses</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>No</td>
<td><strong>Yes</strong> but genus-dependent for other paramyxoviruses</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>DNA / RNA genome</td>
<td>dsRNA, segmented</td>
<td>ssRNA(-), unsegmented</td>
<td>reverse transcribing. Genomic nucleic acid is subfamily-dependent: dsDNA for Spumaretrovirinae and ssRNA(+) for Orthoretrovirinae</td>
<td>dsDNA</td>
</tr>
</tbody>
</table>

Table 5.7 The chemical characteristics of orthoreoviruses (Chappell et al., 2005), ferlaviruses (Richter et al., 1996), retroviruses (Linial et al., 2005) and herpesviruses (Davison et al., 2005). Unique traits are bolded and underlined.

Enveloped viruses, as a general rule, are more environmentally labile than unenveloped viruses and this difference in lability has been exploited experimentally to suggest the presence or absence of a viral envelope. Such experiments include exposure to detergents/lipid solvents (e.g. chloroform), high temperatures (e.g. 560°C) and acidic conditions (e.g. pH 3). Of the four viral groups mentioned in Table 5.7, only reoviruses are unenveloped so it would be expected that the viral titre of a reovirus would be the least affected after exposure to these adverse conditions.

Of the four viruses listed in Table 5.7, only herpesviruses and the Spumaretrovirinae subfamily of the retroviruses have DNA genomes and only orthoreoviruses have double-stranded segmented RNA genomes. The ability of a DNA or reverse transcribing (e.g. retrovirus) virus to

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These viruses can sometimes be released from the host cell through the process of budding. In this event, an unstable lipid envelope transiently surrounds the virion (Mertens et al., 2005).
replicate will theoretically be impaired in the presence of a DNA synthesis inhibitor (e.g. 5’ bromo-3’ deoxyuridine [BUDR]), whereas the replication of non-reverse transcribing RNA viruses will be unaffected. However, the situation in retroviruses may not be so straightforward. As an example, syncytial cell formation associated with Bovine leukemia virus was observed despite the presence of a DNA synthesis inhibitor (Graves and Jones, 1981). But in summary, if the viral titre of BHP1-Lung was unaffected by the presence of a DNA synthesis inhibitor, this would be consistent with a reovirus or a paramyxovirus.

Reoviruses, unlike paramyxoviruses, retroviruses and herpesviruses, have a segmented genome that can be electrophoretically (e.g. polyacrylamide gel electrophoresis [PAGE]) separated into a unique arrangement of segments (Giordano et al., 1997). This method has been utilised to positively identify reptilian reoviruses (Ahne et al., 1987b; Vieler et al., 1994; Lamirande et al., 1999).

Acridine orange is a histological stain that stains green in the presence of double-stranded nucleic acid and stains red in the presence of single-stranded nucleic acid (McMaster and Carmichael, 1977) and so green cytoplasmic staining would suggest the presence of a reovirus (Ahne et al., 1987b). Double stranded viral genomes will also exist for herpesviruses and retroviruses but in these two cases, the replication cycle predominantly occurs in the nucleus (Davison et al., 2005; Linial et al., 2005) and so cytoplasmic staining would be minimal. Although not performed on cells infected with a reptilian paramyxovirus, acridine orange would be expected to stain red in both the cytoplasm and the nucleus (Lamb et al., 2005).

Many of these chemical tests have been applied to the reptilian viruses of interest: ferlaviruses (Richter et al., 1996; Marschang et al., 2002), snake reoviruses (Ahne et al., 1987b; Vieler et al., 1994; Blahak, 1995) and herpesviruses (but only in chelonians) (Marschang et al., 2001a). As expected, snake reoviruses showed resistance to lipid solvents and a DNA synthesis inhibitor;
ferlaviruses were resistant to a DNA synthesis inhibitor but susceptible to lipid solvents; and chelonian herpesviruses were susceptible to both DNA synthesis inhibitors and lipid solvents. The author could not find similar investigations assessing reptilian retroviruses, but a report does exist for a retrovirus-like virus in salmon that surprisingly was resistant to both DNA synthesis inhibitors and lipid solvents (Oh et al., 1995).

In summary, utilising a series of chemical experiments can provide several opportunities to obtain valuable biological data about a viral isolate of unknown identity. To this end, BHP1-Lung was analysed using some of these methods.

**Effect of Chloroform and Ether**

BHP1-Lung was exposed to several adverse conditions and after exposure to each, any change in viral titre was recorded (Table 5.8). *Equine herpes virus* (EHV), an enveloped DNA virus grown in a rabbit kidney cell line (RK-13, American Type Culture Collection [ATCC] CCL-37), and *Nelson Bay orthoreovirus* (NBV), an unenveloped RNA reovirus grown in a porcine squamous-equine kidney cell line (PSEK), were used as positive controls. By using these two viruses as controls, these investigations would have the potential to reveal two biological traits about BHP1-Lung: the presence or absence of a lipid envelope and the presence of a DNA or RNA genome.

For each of the following experiments, four to six replicates of each viral isolate were treated and an additional four to six replicates were left untreated.

All three isolates (EHV, NBV and BHP1-Lung) were exposed to chloroform at a final concentration of 33% (v/v) (i.e. one part chloroform to two parts viral isolate) at room temperature for ten minutes. Solutions were then centrifuged at 2,000g for ten minutes at room temperature and the aqueous phase (the supernatant) was collected. Nitrogen was bubbled through the solution to remove residual solvent. These chloroform-treated solutions
were then assessed for viral titre \((\textbf{Table 5.8, experiment one})\) using the Reed-Muench method described earlier \((\textbf{section 4.2.1 – Cell culture})\). Aliquots of NBV, EHV and BHP1-Lung were left untreated to serve as negative controls.

Unexpectedly, the viral titre of NBV was reduced by a factor of 631. This is inconsistent with the results of other reovirus studies where the infectivity of snake reoviruses was resistant to the effects of chloroform \((\text{Ahne et al., 1987b; Vieler et al., 1994; Blahak, 1995})\). However, the effect on viral titre was not quantified in these studies leaving the definition of “resistant” to be subjectively interpreted. Further investigation of the literature revealed that some reoviruses are sensitive to the effects of chloroform (possibly due to denaturation of the protein coat) \((\text{Feinstone et al., 1983})\) but are reliably resistant to the effects of ether \((\text{Engler and Broome, 1969})\). For this reason, treatment of BHP1-Lung and further treatment of NBV and EHV involved exposure to diethyl ether and a lower concentration of chloroform \((10\% \text{ compared to } 33\%)\).

NBV and EHV were exposed to 10\% \((v/v)\) chloroform \((\text{i.e. one part chloroform to nine parts viral isolate})\) and all three isolates were treated with 20\% \((v/v)\) diethyl ether \((\text{i.e. one part ether to four parts viral isolate})\). BHP1-Lung was not exposed to the second concentration of chloroform because it would be unlikely to provide additional information due to the low titre. Exposure to 10\% chloroform and 20\% ether was for ten minutes at 4\(^\circ\)C and overnight at 4\(^\circ\)C respectively. As before, lipid solvents were removed by clarification and bubbling nitrogen gas through each solution. Viral titres were then calculated \((\textbf{Table 5.8, experiments one and two})\).

\textbf{Effect of Acid}

Each isolate was exposed to an acidic environment by mixing one part of virus solution with nine parts of acidified cell culture media \((\text{pH } = 3, \text{ using hydrochloric acid})\) at 4\(^\circ\)C. After incubation at room temperature for 30 minutes, the pH was returned to neutral \((\text{according to the colour of the pH indicator, phenol red})\) using sodium hydroxide. The viral titre was then
determined for each of the three isolates (Table 5.8, experiment one). Several hours after the virus solutions were inoculated onto their respective cell lines, it was noticed that the phenol red pH indicator in each flask was yellowy-orange, indicating that the media was acidic.

Unexpectedly, the viral titre of EHV decreased by a factor of 10 (by comparison the titre of the unenveloped virus, NBV decreased by a factor of 126). Consultation with the literature revealed that Pacheco’s disease virus (PDV-1, an avian herpesvirus) is relatively resistant to acidic environments (TCID$_{50}$ decreased by a factor of 6.3 after exposure to pH = 3) (Cho and McDonald, 1980), while pseudorabies virus (a porcine herpesvirus) is more labile to extreme acidic environments (no infectivity detected at pH = 3) (Zhu et al., 2011). For experiment two (Table 5.8), isolates were exposed to acid (pH = 3) overnight at 4°C and afterwards, cell culture media was alkalinised to a pH of 8.5 (using sodium hydroxide and a pH meter).

**Effect of a DNA synthesis Inhibitor**

For DNA synthesis inhibition, 5’ bromo-3’ deoxyuridine (BUDR; Sigma-Aldrich, cat. no. B5002-100MG; reconstituted in water), was added to cell culture medium at a final concentration of 100µg/mL and left overnight at 4°C to incubate. Viral titre was then determined as before.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Untreated</th>
<th>Chloroform</th>
<th>Ether (20% v/v)</th>
<th>Acid (pH = 3)</th>
<th>5’ Bromo-3’ deoxyuridine (BUDR, 100µg / mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment One</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Equine herpes virus</em></td>
<td>$10^{6.3}$</td>
<td>$&lt; 10^{2.1}$</td>
<td>&gt; 15,849</td>
<td>n.d.</td>
<td>$10^{5.3}$</td>
</tr>
<tr>
<td><em>Nelson Bay orthoreovirus</em></td>
<td>$10^{6.3}$</td>
<td>$10^{3.5}$</td>
<td>631</td>
<td>n.d.</td>
<td>$10^2$</td>
</tr>
<tr>
<td><em>BHP1-Lung</em></td>
<td>$10^{2.7}$</td>
<td>$&lt; 10^{2.1}$</td>
<td>&gt; 4</td>
<td>$&lt; 10^{3.5}$</td>
<td>$10^{2.7}$</td>
</tr>
<tr>
<td><strong>Experiment Two</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Equine herpes virus</em></td>
<td>$10^{5.1}$</td>
<td>$&lt; 10^{2.1}$</td>
<td>&gt; 1,000</td>
<td>$&lt; 10^{2.2}$</td>
<td>8</td>
</tr>
<tr>
<td><em>Nelson Bay orthoreovirus</em></td>
<td>$10^{7.1}$</td>
<td>$10^{1.8}$</td>
<td>200</td>
<td>$10^6.5$</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 5.8 Effect of ether, acid, a DNA synthesis inhibitor and two concentrations of chloroform on the viral titre (TCID$_{50}$) of *Equine herpes virus* (EHV), an enveloped DNA virus, *Nelson Bay orthoreovirus* (NBV), an unenveloped RNA virus and BHP1-Lung. **Experiment One:** All three isolates were exposed to 33% (v/v) chloroform. One part of virus solution was exposed to nine parts of cell culture media with a pH of 3 and then returned to a neutral pH. The DNA synthesis inhibitor, 5’ bromo-3’ deoxyuridine (BUDR), was added to each isolate to a final concentration in the flask of 100µg/mL. Only BHP1-Lung was exposed to ether. **Experiment Two:** Only NBV and EHV were analysed in this experiment. These two viruses were exposed to a lower concentration of chloroform (10% v/v), ether (20%), acid (pH = 3) and the DNA synthesis inhibitor (BUDR) using the methods of experiment one.
Discussion of Results of Chemical Experiments

In this series of experiments, a number of unexpected results emerged: (1) the infectivity of *Equine herpes virus* (EHV, an enveloped virus), was found to be more resistant to treatment with an acidic solution than *Nelson Bay orthoreovirus* (NBV, an unenveloped virus); (2) when these two isolates were exposed to a DNA synthesis inhibitor, the infectivity of EHV consistently fell below the lower limit of detection but in the second experiment only, the infectivity of NBV decreased by a factor of 3,162, inconsistent with the presence of an RNA genome; (3) the infectivity of EHV was substantially reduced after exposure to chloroform and diethyl ether while for NBV, the infectivity was reduced after exposure to chloroform but was relatively unaffected by exposure to ether. Although these results were found to be initially surprising, all the observations (with the exception of the decrease in infectivity of NBV after the second exposure to BUDR) have been reported elsewhere in the literature (see earlier).

These experiments were only able to provide a limited amount of information about the biological traits of BHP1-Lung. While the infectivity of BHP1-Lung was reduced after exposure to chloroform, the untreated viral titre was not high enough to assess the full extent of this decrease. The infectivity of BHP1-Lung after exposure to diethyl ether could not be assessed because residual ether (not completely eliminated by nitrogen gas) affected the viper heart cells even at dilutions that were greater than the untreated viral titre. Put another way, due to the effects that diethyl ether had on the viper heart cells, only titres (TCID$_{50}$) of BHP1-Lung that exceeded $10^{3.5}$ mL$^{-1}$ would be detectable. The untreated TCID$_{50}$ of BHP1-Lung was $10^{2.75}$ mL$^{-1}$.

Similarly, the effect that treating the isolate with acid had on the viper heart cells limited the lower limit of detection to a TCID$_{50}$ of $10^{3}$ mL$^{-1}$, again greater than the untreated TCID$_{50}$ of BHP1-Lung.

Future studies that investigate the chemical resistance of BHP1-Lung should attempt to improve the untreated titre of the isolate and ensure that even trace amounts of residual
chloroform, ether and acid are removed before exposure to host cells. As mentioned earlier (page 158), in the hands of another virologist, a TCID$_{50}$ for BHP1-Lung of at least 10$^5$ mL$^{-1}$ was seen. Attempts to adapt BHP1-Lung to an alternate cell line may have yielded a greater viral titre. In other species of virus, viral titres have been increased through the use of serial passage (Churchill et al., 1969; Petkova et al., 1986).

In contrast to these findings, the infectivity of BHP1-Lung was unaffected by the presence of a DNA synthesis inhibitor (BUDR). Although NBV showed a significant decrease in infectivity after exposure to BUDR in the second experiment (Table 5.8), it was still detectable. In contrast, the infectivity of EHV fell below the lower limit of detection in both experiments. These experiments are consistent with BHP1-Lung containing an RNA genome. Retroviruses rely on reverse transcribing their RNA genome into proviral DNA (Linial et al., 2005) and so it would be theoretically expected that the infectivity of these viruses would be eliminated (or at least considerably reduced) by a DNA synthesis inhibitor. However, experimental data on Bovine leukemia virus and a retrovirus-like virus, suggests this may not be a reliable trait inherent to all retroviruses (Graves and Jones, 1981; Oh et al., 1995).

There are physical experiments and other chemical experiments that could have been utilised in these studies. As an example of a physical experiment, the effect of filtration on viral titre could have been analysed. Observing cytopathic effects after passing a solution of BHP1-Lung through a syringe-tip filter (e.g. 0.45µm, 0.2µm and/or 0.1µm pore-size diameters) may have shown that BHP1-Lung contained a filterable agent consistent with a virus (as opposed to other non-filterable microbes) but based on the low titre of BHP1-Lung and the variability in size of the four syncytium-forming viruses (reoviruses, retroviruses, herpesviruses and especially paramyxoviruses) it was unlikely to be of any further benefit and is not commonly reported in the literature. In 1995, Blahak reported that the haemagglutinating ability and infectious titre of a ferlaviral isolate was significantly decreased after exposure to $56^0$C; and so
susceptibility to heat would form another physicochemical experiment that could have been performed on BHP1-Lung. In contrast to ferlaviruses, orthoreoviruses (all currently described reptilian reoviruses) are able to withstand extremes in heat (up to 55°C) (Chappell et al., 2005). However, in light of the low titre of BHP1-Lung, this test was not pursued.

Examining cells infected with BHP1-Lung under a fluorescent microscope after staining with acridine orange could have provided some further insight into the genome of this virus. Attempts were made to do this with BHP1-Lung-infected viper heart cells but in the author’s hands, the stain rapidly deteriorated after exposure to fluorescent light, preventing accurate assessment of the monolayer (data not shown). Staining iguana heart (IgH2) cells infected with a snake orthoreoviruses with acridine orange has been used with success by other authors (Ahne et al., 1987b).

Extractions of dsRNA from samples of BHP1-Lung could have been electrophoretically separated in an attempt to visualise a segmented genome that, amongst reoviruses, herpesviruses, retroviruses and paramyxoviruses, is unique to the reoviruses. The electropherotype of reptilian orthoreoviruses has been identified by other authors (Ahne et al., 1987b; Vieler et al., 1994; Blahak, 1995) and attempts to identify it in this study should have been made. Again, the low titre of BHP1-Lung may have prevented identification.

Finally, taking aliquots of BHP1-Lung, ultracentrifuging those through a density gradient of sucrose or caesium chloride and then testing the viral titre of sequential fractions of the various densities may have provided an insight into the buoyant density of the isolate. Specific densities have been reported for the four virus groups of interest (Table 5.5).

In summary, the results of the chemical experiments performed on BHP1-Lung were mostly equivocal and this was best explained by the low viral titre. The presence of a DNA synthesis
inhibitor did not affect the viral titre of BHP1-Lung and this suggests the presence of an RNA genome.

5.3.6 Polymerase Chain Reaction

BHP1-Lung was tested by an assortment of methods (e.g. haemagglutination and neuraminidase activity, electron microscopic examination etc) but the most time and effort was committed to testing BHP1-Lung by polymerase chain reaction (PCR). Although PCR required background knowledge about BHP1-Lung, its lower limit of detection may have been able to overcome the low viral titre that had questioned many of the results previously obtained. PCRs were performed in accordance with the methods described earlier (section 4.8 – Polymerase Chain Reaction).

Initially, efforts were primarily focussed on screening BHP1-Lung with a wide variety of ferlavirus primers (Tables 4.3 and 4.4). No amplicons of the expected size were generated. Next, primers that had the capability to detect a broader range of paramyxoviruses were tested on BHP1-Lung (Table 4.3). The hemi-nested pan-Pneumovirinae PCR primer set published by Tong et al. (2008) (PNE-R, PNE-F1 and PNE-F2) produced an amplicon close to the expected size but sequencing and searching GenBank (National Center for Biotechnology Information, Bethesda, Maryland) using BLASTN (Basic Local Alignment Search Tool for Nucleotides, http://blast.ncbi.nlm.nih.gov/Blast.cgi) could not confirm that the PCR product was viral in origin. No other paramyxoviral PCR primer set produced an amplicon near to the expected size.

As more and more results emerged from the other experiments being concurrently performed on BHP1-Lung, increasing validity was given to the possibility that BHP1-Lung may represent a reovirus. A reptilian reovirus would be explanatory, or at least consistent, with (1) the low grade respiratory disease and stomatitis seen in the host (a black-headed python) during an outbreak of neurorespiratory disease, (2) the syncytial cell formation in cell culture, (3) the
apparent absence of haemagglutinating and neuraminidase activity, (4) the inability to detect anti-ferlaviral antibodies in the host and (5) the negative PCR results for the presence of a paramyxovirus. For these reasons, PCR primers were selected from the literature, and others were designed in-house, that could theoretically detect a wide range of reoviruses (Table 4.5). Some of these primer sets contained primers that could be used in various permutations to provide additional opportunities to detect a reovirus. The reovirus primers designed by the author (A-F, B-F, C-F, B-R, C-R and D-R) and the primer sets published by Wellehan et al. (2009), Landolfi et al. (2010) and Leary et al. (2002) were used in every permutation that would theoretically generate an amplicon. Despite these attempts, no amplicons of the expected size were produced.

Investigating the possibility that BHP1-Lung was a retrovirus was to be initially postponed for reasons already mentioned (section 5.2.6 – Polymerase Chain Reaction). Briefly, it would be difficult to differentiate an endogenous from an exogenous retrovirus and the link between retroviruses and snake disease has not always been clear (Huder et al., 2002; Vancraeynest et al., 2006; Chang and Jacobson, 2010; Hofacre and Fan, 2010).

The histories, clinical signs and pathological findings of the animals in the Queensland collection that produced BHP1-Lung were inconsistent with an adenovirus or herpesvirus infection but broadly-reactive PCRs for the detection of these two virus families were available (Table 4.5). Remaining open to a serendipitous discovery, BHP1-Lung was tested for the presence of these two virus families but in both cases the results were negative.

Months spent testing BHP1-Lung for the presence of paramyxoviruses and reoviruses had failed to identify a virus within this sample. Although the author had access to, and utilised,

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8 During the adenovirus testing of BHP1-Lung, samples from lizards with histopathology consistent with an adenovirus infection were screened using the primer set described by Wellehan et al. (2004) and adenoviruses were detected. These results were published as the first detection of reptilian adenoviruses in Australia (see Appendix for manuscript).
positive control material for these PCRs (section 4.8 – Polymerase Chain Reaction), an ongoing concern was that the negative testing of BHP1-Lung could be attributed to technical reasons i.e. were negative results true or false? To this end, the Jacobson Laboratory at the University of Florida and Dr Rachel Marschang at the University of Hohenheim were both approached about testing BHP1-Lung for ferlavirus and reovirus by PCR using the methods utilised in their diagnostic services. While the importation of these samples into the United States of America was bureaucratically prohibitive, importing the samples into Germany was likely to be possible. Before pursuing this any further it was decided to analyse BHP1-Lung using high-throughput sequencing (section 6.1 – High-throughput Sequencing).

5.4 Chapter Summary

Snake samples were received from most Australian states and territories. Samples were submitted if there was a suspicion that the snake was infected with a pathogenic virus. Samples were obtained from snakes with histories, clinical signs, overseas diagnostic test results and/or pathological findings that were consistent with a viral infection affecting the neurorespiratory systems. Some submissions were from snakes that were in-contact with affected snakes. In total, 463 samples were received and 217 were analysed for haemagglutination activity, 5 for neuraminidase activity, 86 for the presence of anti-ferlaviral antibodies using a haemagglutination inhibition assay, 50 were tested by polymerase chain reaction and 161 were inoculated onto cell culture in an effort to isolate a virus. In addition to this, a small selection of samples were analysed by electron microscopy.

Some of these tests were complicated by confounding variables but ultimately, virus isolation provided an important result. Viruses were isolated from nine samples from five snakes. In all cases, the cytopathic effects seen in cell culture consisted primarily of syncytial cell formation in the absence of significant cell lysis and based on this, it was decided that these viruses were most likely to be from one of four families: Paramyxoviridae, Reoviridae, Retroviridae or
Herpesviridae. The virus obtained from the lung suspension of a black-headed python (Aspidites melanocephalus) was named BHP1-Lung and was tested by several methods in an effort to determine the virus family it belongs to. Results of some of these tests were complicated by the low viral titre of this isolate. However, it seemed likely that BHP1-Lung possessed an RNA genome. Extensive testing by polymerase chain reaction for the presence of a paramyxovirus, reovirus, adenovirus and herpesvirus was unable to identify the virus family that BHP1-Lung belongs to.

The next chapter describes how high-throughput sequencing was employed to determine the identity of BHP1-Lung.
Chapter 6 - Molecular Identification of BHP1-Lung as a Novel Paramyxovirus Named Sunshine virus

This chapter describes the use of high-throughput sequencing to identify BHP1-Lung as a novel paramyxovirus. BHP1-Lung was named Sunshine virus as homage to the geographical origin of the first isolate: the Sunshine Coast of Queensland, Australia. The chapter is divided into six sections. In the first section (6.1 - High-throughput Sequencing), the justification for using high-throughput sequencing is explained followed by the series of steps that were taken to analyse the considerable volume of sequence data. This section concludes after BHP1-Lung had been confidently identified as a paramyxovirus. In the next section (6.2 - Partial Genome Characterisation), the open reading frames of BHP1-Lung are analysed and compared to other paramyxoviruses. Particular attention is focussed on the attachment gene in an effort to clarify the ambiguous test results obtained from earlier phenotypic testing (e.g. haemagglutination and neuraminidase assays).

The first two sections confirm that BHP1-Lung is a unique paramyxovirus and so the third section (6.3 – Proposing the Name Sunshine virus for BHP1-Lung) justifies the name that was selected for it. In the fourth section (6.4 - Sunshine virus Phylogeny), the phylogenetic work of Dr Jim Wellehan is reproduced that places Sunshine virus within the family Paramyxoviridae but outside of the two existing subfamilies. In section five (6.5 – Development and Validation of a Sunshine virus Polymerase Chain Reaction Primer Set), the primers that were used unsuccessfully for the detection of a paramyxovirus in BHP1-Lung are retrospectively analysed for their suitability to detect Sunshine virus. They were found to be unsuitable and so new primers were designed with the capability to screen various sample types for the presence of Sunshine virus. The chapter concludes with a summary of the findings presented in the previous five sections (6.6 – Chapter Summary).
6.1 High-throughput Sequencing

At this point of the project, at least one virus had been isolated from five snakes (Table 5.3) but extensive testing on one of these isolates, BHP1-Lung, by polymerase chain reaction (PCR) had failed to reveal the identity of the virus. The decision was then made to test this sample by high-throughput sequencing. Unlike PCR, which relies on primers annealing to complementary areas on a target sequence, high-throughput sequencing does not depend on any prior knowledge about the target sequences. This gives it a distinct advantage compared to PCR. Unbiased sequence-independent detection has the ability to detect novel viruses and has already been used successfully in several species of veterinary significance: pigs, parrots, turtles, minks, bees, bats and monkeys (Kreuze et al., 2009). After some preliminary enquiries, a Swiss commercial company, Fasteris, was found that could tailor their processing methods to the requirements of this sample.

Once it was decided that high-throughput sequencing would be utilised, the next decision that needed to be made was whether RNA or DNA would be submitted to Fasteris. The titre (TCID\textsubscript{50}) of BHP1-Lung had remained unchanged in the presence of a DNA synthesis inhibitor (BUDR, see Table 5.8) suggesting the presence of an RNA genome (e.g. paramyxovirus, reovirus). Selecting RNA as the template for high-throughput sequencing would be a necessity for non-reverse transcribing RNA viruses (because DNA is not synthesised during their replication) but it would also be able to detect the viral transcripts and genome of reverse transcribing RNA viruses (e.g. retroviruses) (Nakamura et al., 2009) and the viral transcripts of DNA viruses (e.g. herpesviruses) (Nakamura et al., 2009; Ma et al., 2011).

To process RNA samples obtained from cellular extracts, Fasteris usually removes abundant ribosomal RNA (rRNA) from total RNA by selectively extracting polyadenylated RNA species (e.g. mRNA) using oligo(dT) purification. It has been estimated that rRNA constitutes approximately 90% of all cellular RNA, so removing it would improve the likelihood of
detecting viral RNA (He et al., 2010). But considering some viruses, such as paramyxoviruses, are not polyadenylated at the 3’ end of their genome (Lamb et al., 2005), this purification step would need to be skipped. After processing, Fasteris would then perform Illumina® sequencing.

Figure 6.1 provides an overview of the extraction of total RNA from viper heart cells (VH2) infected with BHP1-Lung, to the submission to Fasteris for further processing, and finally, to the de novo assembly of unique contigs.

RNA Extraction from BHP1-Lung-infected VH2 Cells

Briefly, RNA was extracted from a 25cm² flask of BHP1-Lung-infected VH2 cells, which was displaying extensive cytopathic effects (CPE). Media was removed and the monolayer was rinsed with phosphate-buffered saline (PBS, 130mM NaCl, 7mM KH₂PO₄, 3mM Na₂HPO₄·2H₂O, pH 7.4). 1mL of Trizol® LS (Invitrogen, Mulgrave, Victoria, cat. no. 10296-028) was added to the flask, mixed thoroughly with the cell lysate that it created, and then transferred to a new tube. 333µL of chloroform was added, the tube was vortexed and then left at room temperature for five minutes. The contents of the tube were then transferred to a phase lock gel heavy separator tube and centrifuged at 12,000g for 15 minutes at 4°C. The volume of clear supernatant was then quantified and mixed with 100% ethanol to a final ethanol concentration of 33% (v/v). This mixture was then added to an RNeasy® spin column (Qiagen, Doncaster, Victoria, cat. no. 74104) and centrifuged at 8,000g for one minute. Flow through was discarded and the remaining washes were performed in accordance with the manufacturer’s instructions. RNA was eluted into 60µL of RNase-free water. Ribosomal RNA (rRNA) was then twice removed from total RNA using the Ribominus™ Eukaryote Kit (Invitrogen, Mulgrave, Victoria, cat. no. A10837-02). The concentration and purity of RNA was determined spectrophotometrically (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, Delaware): 249ng/µL, 260nm : 280nm absorbance = 2.14, 260nm : 230nm absorbance = 2.30. rRNA-depleted RNA was then adsorbed to the silica membrane of a spin
column from the Ribominus™ Concentration Module (Invitrogen, Mulgrave, Victoria, cat. no. K1550-05). This column was then stored under ambient conditions for one week before being eluted. Repeat analysis by spectrophotometry ensured there had not been any significant deterioration during storage. This extraction procedure (now validated) was then repeated on another flask of BHP1-Lung-infected cells and the rRNA-depleted RNA was adsorbed to another silica membrane before being sent to Fasteris in Switzerland, under ambient conditions, for elution and further processing.

**Processing, Sequencing and de novo Assembly Performed by Fasteris**

Fasteris cleaved the RNA into small pieces (size not specified) using zinc. Single strand cDNA (i.e. RNA:cDNA hybrids) was then synthesised using reverse transcriptase and random hexamers. This was followed by second strand cDNA synthesis using DNA polymerase and RNase H. Overhangs were converted to blunt ends using T4 DNA polymerase and Klenow DNA polymerase. The exonuclease activity of these enzymes removes 3’ end overhangs and the polymerase activity fills in the 5’ overhangs. The 3’ ends were then polyadenylated to create binding sites for oligonucleotide adaptors, which were ligated to the cDNA fragment ends. cDNA was then purified and size-enriched (200bp +/- 25bp) using agarose gel electrophoresis followed by gel extraction. cDNA was amplified using two primers that annealed to the end adaptors. The cDNA library was then sequenced using a Genome Analyser GAIIx (Illumina®). In total, 10,544,936 reads (each 38 nucleotides long) were sequenced and de novo assembly produced 5,818 unique contigs.
Figure 6.1 Sequencing and assembling the rRNA-depleted total RNA from viper heart (VH2) cells infected with BHP1-Lung. Total RNA was extracted from infected cells and then depleted of ribosomal RNA (rRNA). From this, 10,544,936 reads were sequenced and from these, 5,818 unique contigs could be assembled. An assembled contig that is 69 nucleotides long is provided as an example. The minimum coverage of this 69-nucleotide contig is one, the maximum coverage is all 36 contigs, the mean coverage is 19.8 and the standard deviation of coverage is 12.5.
**Analysis of Data**

**Figure 6.2** outlines the next series of steps that were performed to analyse these unique contigs. The Basic Local Alignment Search Tool (BLAST; [http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) was used to try and determine the identity of these unique sequences. Batch searches were performed and the results downloaded. Results were then searched for the word “virus”. From the 5,818 unique contigs, 326 had BLAST hits for the word “virus”. The viral family was then determined for each BLAST hit and viruses not known to occur in vertebrates were excluded. This excluded host DNA (e.g. virus receptors) and viruses of algae, invertebrates, plants, protozoa and fungi. This left 212 unique contigs representing 25 classified families and one unclassified virus (Salterprovirus). Next, ORF Finder ([http://www.ncbi.nlm.nih.gov/projects/gorf/](http://www.ncbi.nlm.nih.gov/projects/gorf/)) was used to identify open reading frames (ORFs) and then search the protein database of GenBank (National Center for Biotechnology Information, Bethesda, Maryland) for similarities. Only one, an 11,709 nucleotide contig, showed strong similarity to a virus. This sequence had one ORF with a predicted amino acid sequence that was similar to the fusion glycoprotein superfamily and a second that was similar to paramyxovirus RNA-dependent RNA-polymerase.
Figure 6.2 Analysis of 5,818 unique assembled contigs to identify a single contig with strong similarity to a paramyxovirus. By searching all 5,818 unique contigs with the Basic Local Alignment Search Tool for Nucleotides (BLASTN), 326 were identified with search results that contained the word "virus". 114 were excluded on the basis that they were viruses that occur in non-vertebrates (algae, plants, invertebrates, fungi and protozoa) or were of host DNA origin (e.g. virus receptors). Only one of the remaining contigs contained open reading frames (ORFs) with strong similarities to a paramyxovirus.
The raw sequencing data (the 10,544,936 reads) were then reassembled “in-house” using CLC Genomics Workbench® software (CLC Bio®) to look for additional sequence information belonging to the putative paramyxoviral genome. A 17,187 nucleotide contig was assembled from 292,587 reads (2.77% of total reads) that extended the 11,709 nucleotide contig, that had been assembled by Fasteris, by an additional 5,478 nucleotides at the 3’ end of the viral genome. These additional nucleotides contained ORFs with strong similarities to paramyxoviral matrix (M) and nucleocapsid (N) ORFs. The mean coverage of this 17,187 nucleotide contig was 648 reads with a standard deviation of 605 reads. There was a minimum of one read and a maximum of 4,647 reads over the length of the contig (Figure 6.1 provides an example to clarify the definitions of these terms). The first 21 nucleotides from the 3’ end had 16 or fewer reads, while the last four nucleotides at the 5’ end, had four or fewer reads. The remaining 17,162 nucleotides (of the 17,187 nucleotide contig) had 20 or more reads contributing to each nucleotide of this contig.

This data showed strong evidence for the presence of a paramyxovirus in viper heart cells (VH2) infected with BHP1-Lung. This result was also explanatory for the cytopathic effects (CPE) seen in this flask (primarily syncytial cell formation). At this point, it was apparent that two lines of investigation were needed. Firstly, what was this new paramyxovirus? Was it another ferlavirus, or was it unique amongst the paramyxoviruses? Further characterisation of the genome and phylogenetic placement of this virus would be used in an attempt to answer these questions. Secondly, what is the significance of this finding? Cost restraints had prevented the analysis of an RNA sample of uninfected VH2 cells so the possibility that this paramyxovirus was endogenous to the VH2 cells that had been used in this project, could not be excluded. To investigate this possibility, PCR primers were designed that could screen tissue samples and uninfected cells for this paramyxovirus. The next four sections concern themselves with partially characterising the genome of this paramyxovirus, proposing a name.
for it, phylogenetically placing it in the paramyxovirus family and designing and using PCR primers to investigate the significance of this new virus.

### 6.2 Partial Genome Characterisation

Figure 6.3 maps the paramyxovirus genome that is present in the 17,187 nucleotide contig. The predicted amino acid sequences from four paramyxoviral open reading frames (ORFs) were detected by BLASTP (Basic Local Alignment Search Tool for Proteins): nucleocapsid (N), matrix (M), fusion (F) and polymerase (L). The order of conservation between paramyxoviral proteins has been previously described (Kurath et al., 2004) (most conserved to least conserved): V-carboxy domain > polymerase (L) > matrix (M) / fusion (F) > nucleocapsid (N) / attachment (HN) > zinc binding protein (V) > phosphoprotein (P); so identifying N, M, F and L from the 17,187 contig using BLASTP was not unexpected. Three other ORFs were present but they could not be identified by BLASTP.
Figure 6.3 Map of genomic RNA (3’ to 5’) of a 17,187 nucleotide length of sequenced genome from viper heart cells infected with BHP1-Lung. Numbers represent nucleotide lengths. ORF = open reading frame. Where GenBank similarity could not be validated, putative annotation has been used based on the ORF that is positioned at the corresponding position of other paramyxoviruses.
The Putative Attachment Protein

Between the fusion and polymerase genes, lies an ORF, 2,031 nucleotides in length. In all paramyxoviruses, except the Pneumovirus genus, this is the location of an ORF that codes for the attachment protein (H, HN or G). GenBank searches using BLASTN (Basic Local Alignment Search Tool for Nucleotides) and BLASTP did not provide any meaningful results so the (putative) attachment protein was aligned with those of 18 paramyxoviruses: two from each of the seven currently accepted genera (Respirovirus, Rubulavirus, Avulavirus, Morbillivirus, Henipavirus, Pneumovirus and Metapneumovirus), one from each of the two newly-proposed genera (Ferlavirus and Aquaparamyxovirus) and two unclassified paramyxoviruses (Menangle virus and Beilong virus). Other authors have presented the relatedness of paramyxoviral attachment proteins in a phylogenetic tree (Li et al., 2006) but these proteins do not align as well as other paramyxoviral proteins and so a tree may not accurately depict relatedness (section 6.4 – Sunshine virus Phylogeny). Amino acid sequences were aligned using CLUSTALW (http://www.genome.jp/tools/clustalw/) (Thompson et al., 1994) and the results of pairwise alignment are presented in Table 6.1. The putative attachment protein of BHP1-Lung was the only paramyxovirus that showed weak alignment (<15%) to all other attachment proteins analysed.
Genus
Attachment
protein
Protein
length (AA)
Avian MPV
Human MPV
Bovine RSV
Human RSV
Beilong
Nipah
Hendra
Measles
Rinderpest
Avian PMV2
NCDV
Mumps
Porcine RV
Menangle
Human PIV
Sendai
Atlantic
salmon PMV
FDLV
BHP1-Lung

Sendai

Human
PIV

Menangle

Ferla.

Atlantic
salmon
PMV
AquaPMV

Respiro

Respiro.

Unclass.

?

HN

HN

HN

HN

HN

HN

HN

HN

HN

H

H

G

G

G

G

676

564

576

575

572

595

576

582

577

583

609

617

604

602

734

9.91
13.69
14.39
11.37
9.32
10.63
11.09
11.51
11.00
9.09
9.88
9.97
9.90
11.60
10.66
10.61

10.28
12.78
12.06
13.71
31.56
19.33
18.44
12.41
13.47
19.33
24.29
19.68
24.64
13.65
34.04
37.06

10.07
13.70
12.06
13.38
27.78
20.31
19.44
11.98
11.11
18.06
19.44
21.18
20.66
14.06
36.01
37.21

9.74
15.52
12.06
12.71
24.87
20.17
18.26
13.22
12.87
18.43
20.35
16.52
19.13
12.87
44.75

9.96
15.07
14.40
13.71
22.73
18.01
17.13
12.76
13.29
16.78
20.45
18.71
20.98
12.59

9.23
12.78
13.62
13.38
11.93
12.94
11.43
10.08
10.08
15.78
15.94
18.9
17.19

10.59
14.61
12.45
11.71
16.84
14.76
14.06
10.59
12.15
27.60
28.99
39.93

10.82
14.15
12.84
12.71
19.93
11.68
11.86
11.34
11.17
25.26
29.12

10.05
13.70
11.28
13.04
20.80
14.04
12.48
11.78
11.44
32.41

9.78
13.70
12.45
11.37
16.30
12.52
10.80
11.15
10.63

11.97
14.15
12.06
11.71
12.31
13.95
13.08
58.78

11.28
11.87
10.89
11.37
12.97
12.79
12.58

10.42
13.70
13.23
13.04
17.55
78.74

9.57
13.70
11.67
13.04
18.27

10.60
12.78
12.06
11.37

11.11

37.59

BHP1Lung

FDLV

Unclass.

11.17
11.17

37.59
11.11

Porcine
RV

Mumps NCDV

Avian
Rinderpest Measles
PMV2


29.12
28.99
15.94
20.45
20.35

32.41
25.26
27.60
15.78
16.78
18.43

Morbilli.

Hendra

Nipah


10.63
11.44
11.17
12.15
10.08
13.29
12.87

58.78
11.15
11.78
11.34
10.59
10.08
12.76
13.22

12.58
13.08
10.80
12.48
11.86
14.06
11.43
17.13
18.26

78.74
12.79
13.95
12.52
14.04
11.68
14.76
12.94
18.01
20.17

Beilong

Human
RSV

Bovine
RSV


Human Avian
MPV
MPV
MPV

MPV

G

G

G

299

257

219

585

17.39
15.98
29.18

15.95
13.24

24.20

18.27
17.55
12.97
12.31
16.30
20.80
19.93
16.84
11.93
22.73
24.87

11.37
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29.18
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12.84
12.45
13.62
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13.24
15.98
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11.87
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13.70
13.70
14.15
14.61
12.78
15.07
15.52

24.20
15.95
17.39
10.60
9.57
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11.28
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9.78
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9.23
9.96
9.74

44.75

12.59
12.87

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16.52

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36.01

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11.98

19.44

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27.78

13.38

12.06

13.70

10.07

37.06
10.61

34.04
10.66

13.65
11.60

24.64
9.90

19.68
9.97

24.29
9.88

19.33
9.09

13.47
11.00

12.41
11.51

18.44
11.09

19.33
10.63

31.56
9.32

13.71
11.37

12.06
14.39

12.78
13.69

10.28
9.91

Table 6.1 Pairwise alignments of the attachment proteins of 19 paramyxoviruses using CLUSTALW. Numbers represent percentage similarities. The similarities of the
attachment proteins of viruses from the same genera are in adjacent columns and highlighted in red. BHP1-Lung has the only attachment protein that has less than a
15% similarity to each of the other 18 proteins. FDLV = Fer-de-Lance virus (GenBank accession number: NC_005084). Atlantic salmon PMV [Paramyxovirus]

206 | P a g e


Despite this analysis, the putative attachment protein of BHP1-Lung remains poorly characterised. Both haemagglutination (HA) and neuraminidase (NA) activity could not be convincingly detected phenotypically (sections 5.3.2 – Haemagglutination and Haemadsorbing Activity and 5.3.3 – Neuraminidase Activity) and the pairwise protein alignments were also non-contributory. While it may therefore be tempting to claim that this protein is a G (glycoprotein) attachment protein (i.e. possesses neither HA or NA activity), the experiments presented in this thesis do not provide enough evidence to support this. Future studies that attempt to clarify the identity of this attachment protein could have direct clinical significance. For example, the other snake paramyxoviruses, the ferlaviruses, have HN attachment proteins which can be used in haemagglutination inhibition (HI) assays to detect anti-ferlaviral antibodies (section 2.3.6 – Haemagglutination Inhibition). To detect the humoral response to paramyxoviruses with G attachment proteins, HI assays cannot be used. Virus neutralisation and/or ELISA (enzyme-linked immunosorbent assay) assays are often used instead (Lamb and Parks, 2007). Analysis of the predicted three-dimensional structure of this uncharacterised protein may provide further insight into its form and function (Langedijk et al., 1997).

**The Putative Phosphoprotein and a 540-nucleotide Open Reading Frame**

A 1,677 nucleotide ORF can be seen 3’ to the matrix (M) ORF. In all paramyxoviruses this is the location for a phosphoprotein (P). A GenBank search using BLASTP and BLASTN was unable to confirm the identity of this putative phosphoprotein. Further analysis of this protein was not pursued.

Additionally, between this putative phosphoprotein (P) and N, lies a 540 nucleotide (179 amino acids) ORF. Only paramyxoviruses from the proposed genus Ferlavirus (all previously described reptilian paramyxoviruses) are known to translate a protein, named “U” for unknown, in this region (Kurath et al., 2004; Marschang et al., 2009). A GenBank search using BLASTP and BLASTN was unable to confirm the identity of this possible ORF. A pairwise alignment of the
amino acid sequences of the U proteins of Fer-de-Lance virus (FDLV, 167 amino acids, GenBank accession number: NP_899655) and another ferlavirus (GonoGer85, 167 amino acids, AY534644) showed an 88.6% similarity to each other but when aligned with the 179 amino acid predicted protein sequence from BHP1-Lung, the pairwise similarities were only 13.2% and 12.6% respectively. Kurath et al. (2004) used electrophoretic separation of ferlaviral proteins to discover that the U ORF translated into a protein. Applying this technique to BHP1-Lung could reveal whether this 540 nucleotide ORF is a protein coding region of the genome. Sunshine virus and the ferlaviruses are not closely related (section 6.4 – Sunshine virus Phylogeny), there is little sequence similarity and therefore no reason to expect that these ORFs are orthologous.

Future characterisations of the Sunshine virus genome could consider analysing the untranslated and intergenic regions (UTRs and IGRs) to look for sequence similarities to other paramyxoviruses. This has been done for other paramyxoviruses (Wang et al., 2000; Li et al., 2006) and may provide some further insight into the biology of Sunshine virus.

In all known paramyxoviruses, the nucleocapsid gene (N) is nearest to the 3’ end and the polymerase gene (L) is nearest to the 5’ end, so it is likely that the sequence information generated from this investigation includes all of the coding regions of this new virus. The depth of coverage from the Illumina® high-throughput sequencing is shallow at both the 3’ and 5’ end of the contig. Therefore, the 17,187 nucleotide contig may not represent the complete genome. 5’ or 3’ rapid amplification of cDNA ends (RACE) may help in defining the terminal ends of this genome (Kurath et al., 2004). The 17,187 nucleotide contig is not divisible by six but without the certainty that this contig represents the complete genome of Sunshine virus, it cannot be concluded whether this new genome conforms to the “rule of six”. The “rule of six” is where the total number of nucleotides in the viral genome is a multiple of six.
(polyhexameric) and is a unique feature of all members of the \textit{Paramyxovirinae} subfamily but not the \textit{Pneumovirinae} subfamily (Kolakofsky \textit{et al.}, 2005; Lamb \textit{et al.}, 2005).

The 17,187 nucleotide contig has been deposited into GenBank under the accession number JN192445.

\section*{6.3 Proposing the Name Sunshine virus for BHP1-Lung}

The Basic Local Alignment Search Tool for Proteins (BLASTP) had revealed four proteins (N, M, F and L) with strong similarity to other paramyxoviruses. Open reading frames (ORFs) that presumably coded for the phosphoprotein (P) and the attachment protein could not be confidently aligned with other paramyxoviruses. These findings supported the conclusion that BHP1-Lung was unique among paramyxoviruses and should be provided with its own name.

Formal recognition of new viral taxa can only be provided by the International Committee on Taxonomy of Viruses (ICTV, 2010). Proposals must be submitted to the ICTV with a justification as to why the new virus should be taxonomically independent of its closest relatives. This has not been done but as the discoverer of this new virus and for the purposes of clarity when discussing this new virus, a name was suggested.

The rules of orthography are listed on the ICTV website (http://ictvonline.org/codeOfVirusClassification_2002.asp). For BHP1-Lung, it was important that its name did not create confusion with the existing cluster of snake paramyxoviruses; the ferlaviruses. Further constraints were that the name should be euphonious (pleasing to the ear). Several names were considered that did not seem appropriate before the name \textit{Sunshine virus} was chosen (Table 6.2).
Possible names for a new paramyxovirus in Australian snakes

<table>
<thead>
<tr>
<th>Name</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snake neurorespiratory paramyxovirus</td>
<td>• Considers the host, predominant clinical signs and virus family.</td>
</tr>
<tr>
<td></td>
<td>• Does not adequately differentiate it from the ferlavirus group.</td>
</tr>
<tr>
<td>Reptilian neurorespiratory paramyxovirus</td>
<td>• As above but allows for a greater diversity of future hosts but could be confusing if it is never found in non-snake reptiles.</td>
</tr>
<tr>
<td>Ophidian paramyxovirus (OPMV)</td>
<td>• More concise but would cause great confusion with the published literature that refers to ferlaviruses as ophidian paramyxoviruses (OPMV).</td>
</tr>
<tr>
<td>Aspidites virus</td>
<td>• Like Fer-de-Lance virus (FDLV, the type species of the proposed genus Ferlavirus and named after the Fer-de-Lance viper mentioned in the first report of this virus), would be homage to the first species (in this case genus) that the virus was isolated from.</td>
</tr>
<tr>
<td>Australian snake paramyxovirus</td>
<td>• Considers the geographical origin of the first isolate, the host and the virus family.</td>
</tr>
<tr>
<td>Australian reptile paramyxovirus</td>
<td>• As above but allows for a greater diversity of future hosts but could be confusing if is never found in non-snake reptiles.</td>
</tr>
<tr>
<td>Mortalivirus</td>
<td>• A variation of an existing paramyxoviral genus, Morbillivirus.</td>
</tr>
<tr>
<td></td>
<td>• Future epidemiological data may conclude that the name misleadingly implies a high mortality rate.</td>
</tr>
<tr>
<td></td>
<td>• Name is not very descriptive.</td>
</tr>
<tr>
<td></td>
<td>• Breaks the rules of orthography because the word “virus” must be included in the final word of the species name which itself must be more than one word.</td>
</tr>
<tr>
<td>Sunshine virus</td>
<td>• Euphonious and memorable.</td>
</tr>
<tr>
<td></td>
<td>• Refers to the geographical origin of the first isolate which follows a precedent already set by other paramyxoviruses e.g. Menangle virus, Hendra virus, Nipah virus, Sendai virus, Newcastle disease virus, Beilong virus, Mossman virus, Tioman virus and Salem virus.</td>
</tr>
<tr>
<td></td>
<td>• Name could be considered facetious.</td>
</tr>
</tbody>
</table>

Table 6.2 Names that were considered for the new Australian snake paramyxovirus. Ultimately, Sunshine virus was chosen.

Sunshine virus follows a precedent of several other paramyxoviruses whose names were derived from the geographical origin of the first isolate.
6.4 Sunshine virus Phylogeny

The phylogenetic analysis of Sunshine virus was performed by Dr James Wellehan (DVM, MS, PhD, DipACZM, DipACVM) from the University of Florida and formed part of a submitted manuscript of this work. Dr Wellehan has published more than 65 papers predominantly on the infectious diseases of a wide range of animals and his phylogeny methods have been accepted for publication in many journals.

In this section (6.4 - Sunshine virus Phylogeny) work is presented in text boxes that was not performed by the author of this thesis. It is included here nevertheless so that this thesis can contain the most complete account of the current knowledge on Sunshine virus.
Open reading frames (ORFs) were identified using a web-based translator (http://www.vivo.colostate.edu/molkit/translate/index.html). Large ORFs were identified by sequence homology to known paramyxoviruses in GenBank (National Center for Biotechnology Information, Bethesda, Maryland), EMBL (Cambridge, United Kingdom), and Data Bank of Japan (Mishima, Shizuoka, Japan) databases using BLASTP (Altschul et al., 1997), and by location in the genome.

The predicted homologous amino acid sequences of mononegaviral proteins were aligned using MAFFT (Katoh and Toh, 2008). Bayesian analyses of each alignment were performed using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with gamma distributed rate variation and a proportion of invariant sites, and amino acid substitution model jumping. Four chains were run and statistical convergence was assessed by looking at the standard deviation of split frequencies as well as potential scale reduction factors of parameters. The first 10% of 1,000,000 iterations were discarded as a burn in, based on examination of trends of the log probability vs. generation. Two independent Bayesian analyses were run to avoid entrapment on local optima.

Maximum likelihood (ML) analyses of each alignment were performed using PHYLIP (Phylogeny Inference Package, Version 3.66) (Felsenstein, 1989), running each alignment using the program Proml with amino acid substitution models JTT (Jones et al., 1992), PMB (Veerassamy et al., 2003), and PAM (Kosiol and Goldman, 2005) further set with global rearrangements, five replications of random input order, gamma plus invariant rate distributions, and unrooted. The values for the gamma distribution were taken from the Bayesian analysis. Nyamanini virus (GenBank accession number NC012703) was used as the outgroup for the N and L genes, Bornavirus (NC_001607) was used as the outgroup for the M gene, and Atlantic salmon paramyxovirus (EF646380) was designated as the outgroup for the F gene. The alignment was then used to create data subsets for bootstrap analysis to test the strength of the tree
topology (200 re-samplings) (Felsenstein, 1985), which was analyzed using the amino acid substitution model producing the most likely tree in that alignment.

Bayesian phylogenetic analysis of the predicted N and M proteins found that the WAG model of amino acid substitution was most probable with a posterior probability of 1.000 (Whelan and Goldman, 2001). The predicted F protein analysis found the CpRev model to be most probable (posterior probability=1.000), and the predicted L protein analysis found the Blosum model to be most probable (posterior probability=1.000) (Henikoff and Henikoff, 1992; Adachi et al., 2000). Bayesian trees including posterior probabilities of clades are shown (Figures 6.4 - 6.7).
Figure 6.4 Bayesian phylogenetic tree of predicted 1,711-2,331 amino acid sequences of mononegaviral RNA-dependent-RNA polymerase based on MAFFT alignment. Bayesian posterior probabilities of clusters as percentages are in bold, and ML bootstrap values for clusters are given to the right. Nyamanini virus (GenBank accession number NC012703) was used as the outgroup. Sunshine virus is bolded. Thick brackets demarcate viral families, medium brackets indicate paramyxoviral subfamilies, and thin brackets demarcate paramyxoviral genera. Sequences retrieved from GenBank include Bornavirus (GenBank accession # NC_001607), Nyamanini (NC_012703), Duvenhage (EU293119), Flanders (AAN73288), Lake Victoria Marburg (YP_001531159), Sudan Ebola (YP_138527), Newcastle disease virus (NC_002617), Avian Paramyxovirus 2 (HM159993), Avian Paramyxovirus 6 (NC_003043), Human Parainfluenzavirus 2 (NC_003443), Simian Parainfluenzavirus 5 (NC_006430), Mapuera (NC_009489), Porcine Rubulavirus (NC_009640), Mumps (NC_002200), Menangle (NC_007620), Tioman (NC_004074), Beilong (NC_007803), J Virus (NC_007454), Canine Distemper Virus (NC_001921), Dolphin Morbillivirus (NC_005283), Measles (NC_001906), Nipah (NC_002728), Bovine Parainfluenzavirus 3 (NC_002161), Human Parainfluenzavirus 1 (NC_003461), Human Parainfluenzavirus 3 (NC_001796), Sendai (NC_001552), Atlantic Salmon Paramyxovirus (EF646380), Ferlavirus (NC_005084), Avian Metapneumovirus (NC_007652), Human Metapneumovirus (NC_004148), Bovine Respiratory Syncytial Virus (NC_001989), Human Respiratory Syncytial Virus (NC_001781) and Pneumonia Virus of Mice (NC_006579).
Figure 6.5 Bayesian phylogenetic tree of predicted 529-662 amino acid sequences of paramyxoviral fusion proteins based on MAFFT alignment. Bayesian posterior probabilities of clusters as percentages are in bold, and ML bootstrap values for clusters are given to the right. Atlantic salmon paramyxovirus (EF646380) was used as the outgroup. Sunshine virus is bolded. Medium brackets indicate paramyxoviral subfamilies, and thin brackets demarcate paramyxoviral genera. Sequences retrieved from GenBank are from the same genomes as the L genes used in Figure 6.4.
Figure 6.6 Bayesian phylogenetic tree of predicted 142-377 amino acid sequences of mononegaviral matrix proteins based on MAFFT alignment. Multifurcations are marked with arcs. Bayesian posterior probabilities of clusters as percentages are in bold, and ML bootstrap values for clusters are given to the right. Bornavirus (NC_001607) was used as the outgroup. Sunshine virus is bolded. Thick brackets demarcate viral families, medium brackets indicate paramyxoviral subfamilies, and thin brackets demarcate paramyxoviral genera. Sequences retrieved from GenBank are from the same genomes as the L genes used in Figure 6.4.
Figure 6.7 Bayesian phylogenetic tree of predicted 370-738 amino acid sequences of mononegaviral nucleoproteins based on MAFFT alignment. Bayesian posterior probabilities of clusters as percentages are in bold, and ML bootstrap values for clusters are given to the right. Nyamanini virus (GenBank accession number NC012703) was used as the outgroup. Sunshine virus is bolded. Thick brackets demarcate viral families, medium brackets indicate paramyxoviral subfamilies, and thin brackets demarcate paramyxoviral genera. Sequences retrieved from GenBank are from the same genomes as the L genes used in Figure 6.4.
ML analyses of the predicted N, M, and L proteins found the most likely tree resulted from the JTT model of amino acid substitution, and analysis of the predicted F protein found that the most likely tree resulted from the PMB model. These parameters were used for bootstrap analysis. Bootstrap values from ML analysis are shown on the Bayesian trees (Figures 6.4 - 6.7).

None of the analyses found that Sunshine virus clustered within either currently recognized subfamily of the paramyxoviruses. The predicted L gene analysis found that Sunshine virus clustered within the Paramyxoviridae with 100% posterior probability and 100% ML bootstrap value (Figure 6.4). The M gene analysis, while supporting recognized genera as monophyletic, was not able to resolve deeper level relationships, including not forming a distinct monophyletic cluster from the Avulavirus/Rubulavirus and Respirovirus/Henipavirus/Morbillivirus clades in the subfamily Paramyxovirinae. Similarly, the N gene analysis did not resolve deeper level relationships with strong confidence, and actually had weak support for paraphyly of the family Paramyxoviridae, with support in the Bayesian (but not ML) analysis for a clade containing Rhabdoviridae/Paramyxovirinae/Sunshine virus. The M gene analysis found support for genera and subfamilies, but homologous genes are not present in other members of the Mononegavirales to examine deeper level relationships.

We chose to examine amino acid alignments because of concerns regarding non-lineage factors on viral nucleotide composition bias outweighing the true phylogenetic signal. The paramyxoviruses are divergent to the point that the phosphoproteins and attachment proteins cannot be reliably aligned, and this indicates that the phylogenetic signal from synonymous sites is likely to be significantly weakened by homoplasy. While it has been shown that nucleotide alignments may be moderately more informative than amino acid alignments when looking at vertebrate genes (Townsend et al., 2008), viral evolutionary events such as host switches may cause differential biases in different lineages. In the genus Atadenovirus, squamate reptiles appear to be the endemic hosts. These viruses appear to have jumped into
birds and mammals in at least two separate events, and in both cases, host jumps were associated with a large AT bias (Wellehan et al., 2004). Experimental cross-species transmission of a feline lentivirus was shown to have a major impact on nucleotide bias (Poss et al., 2006). Host nucleotide composition and host switches also appear to have a significant impact on astrovirus composition (van Hemert et al., 2007).

Dr Wellehan produced phylogenetic trees based on the entire amino acid sequences of the L, F, M and N paramyxoviral proteins (Figures 6.4 - 6.7). These trees all show that Sunshine virus should be clustered within the family Paramyxoviridae but outside existing subfamilies. All four trees also show that Sunshine virus is related to ferlavirus only at a family level. Previous to the discovery of Sunshine virus, all known reptilian paramyxoviruses had clustered within the proposed genus ferlavirus (Kurath et al., 2004). The paramyxoviruses show considerable host diversity by utilising salmonid, reptilian (birds, snakes, lizards and tortoises), and mammalian hosts, and there is no evidence of host-virus codivergence over large-scale paramyxovirus evolution. Ferlaviruses have been found to infect snakes, lizards and tortoises (Marschang et al., 2009). The use of snake hosts by Sunshine virus is likely an independent event from the use of snake hosts by ferlaviruses.

High-throughput sequencing had generated enough Sunshine virus sequence data to determine that it is a paramyxovirus and subsequent phylogenetic analysis had demonstrated its uniqueness within this family. With the sequence information available, polymerase chain reaction (PCR) primers could be designed that would allow the rapid identification of Sunshine virus in a variety of sample types.
6.5 Development and Validation of a Sunshine virus Polymerase Chain Reaction Primer Set

Illumina® high-throughput sequencing had allowed the identification of a unique paramyxovirus that was subsequently named Sunshine virus. Several PCR primers that had been designed for the detection of a broad range of paramyxoviruses (Tables 4.3 – 4.4) failed to detect Sunshine virus. With the benefit of hindsight, this was not surprising considering the partial genome characterisation (section 6.2 – Partial Genome Characterisation) and phylogenetic assessment (section 6.4 – Sunshine virus Phylogeny) of Sunshine virus both concluded that it was a unique species of paramyxovirus. However, with the recently-available Sunshine virus sequence data, the suitability of these paramyxoviral primers could be analysed in greater detail. It was still possible that the failure of these primers to detect Sunshine virus was technical and not theoretical. Two primer sets of particular interest were chosen for further analysis. Firstly, the L gene primer set described by Ahne et al. (1999b) was chosen because it is used in at least two diagnostic laboratories (University of Florida and University of Hohenheim, Germany) and every ferlaviral isolate that has been tested with these primers has been detected (Marschang et al., 2009). It would be valuable to know if this primer set is unable to detect all known reptilian paramyxoviruses (i.e. all ferlaviruses and Sunshine virus).

The second primer set of particular interest was described by Tong et al. (2008) and had been carefully designed to detect all known members from each of the two paramyxoviral subfamilies: Paramyxovirinae and Pneumovirinae. With Sunshine virus being phylogenetically placed between these two subfamilies (Figures 6.4 – 6.7) it would be interesting to see if it could theoretically be detected by either a pan-Paramyxovirinae or pan-Pneumovirinae set of primers.

To be able to assess the binding sites of the Ahne primers in the ferlavirus and Sunshine virus genomes, the L genes of a ferlaviral isolate, Fer-de-Lance virus (FDLV), and Sunshine virus, were translationally aligned. To do this, the L gene of both viruses was translated into an
amino acid sequence and aligned with CLUSTALW (Thompson et al., 1994). The alignment was cross-referenced with the alignment produced by MUSCLE (Edgar, 2004). Next, the nucleotides from the original gene were reinserted in place of the amino acids. The primer binding sites were found for FDLV, and the analogous region of the Sunshine virus genome was located. These sites were then checked for similarity to the primer sequence (Figure 6.8). The number of bases in the primer that matched with the binding site on the viral genome, both in total, and at the 3’ end of the primer, are presented in Table 6.3. This process was repeated for the primers described by Tong et al. (2008) using the L genes of FDLV and bovine RSV as controls (Figure 6.9 and Table 6.3).
Assessment of the binding affinity of the Ahne L-gene PCR primer set (Ahne et al., 1999b) to Sunshine virus and Fer-de-Lance virus (FDLV). A translational alignment was performed where the L gene nucleotide sequences were translated into amino acid sequences which were then aligned using CLUSTALW (cross-referenced with MUSCLE) and finally, nucleotides were reinserted in place of the amino acids. The four PCR primers (Ahne 5-8) were then positioned into their binding sites to assess their similarity to the corresponding area of the Sunshine virus genome. * = complementary sequence.

<table>
<thead>
<tr>
<th></th>
<th>Sunshine virus (3': 1108-1139)</th>
<th>Ahne 5 (5' → 3')</th>
<th>FDLV (3': 1036-1067)</th>
<th>Sunshine virus (3': 1714-1746)</th>
<th>Ahne 6* (3' → 5')</th>
<th>FDLV (3': 1642-1674)</th>
<th>Sunshine virus (3': 1151-1181)</th>
<th>Ahne 7 (5' → 3')</th>
<th>FDLV (3': 1079-1109)</th>
<th>Sunshine virus (3': 1696-1728)</th>
<th>Ahne 8* (3' → 5')</th>
<th>FDLV (3': 1624-1656)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GATAGGTGGAGCTTTGAGTTGAGTGCAGTGAATTG</td>
<td>GCAGAGTTTTCTTTTTT</td>
<td>GAAATCAGAGTTTTCTTTTTTTAGTG</td>
<td>GCAGAGTTTTCTTTTTTATGTCGA</td>
<td>GCAGAGTTTTCTTTTTTATGTCGA</td>
<td>GCCAGAGTTTTCTTTTTTATGTCGA</td>
<td>CTTATTGGGGTGAGAGGGCTATTTTTAAGG</td>
<td>TAGAGGTTGTTAGCTGTGCA</td>
<td>CGTCTCTAGGAGCTTTTCTTTGCTTGTGATAA</td>
<td>ACCTCTGTCTAGACTTTGGGAAAAATGACTTTG</td>
<td>GCCAGATTATTTCGGCAAAGATG</td>
<td>CAGAGGGCGCATATTGCGAGATGACATAG</td>
</tr>
</tbody>
</table>

Figure 6.8 Assessment of the binding affinity of the Ahne L-gene PCR primer set (Ahne et al., 1999b) to Sunshine virus and Fer-de-Lance virus (FDLV). A translational alignment was performed where the L gene nucleotide sequences were translated into amino acid sequences which were then aligned using CLUSTALW (cross-referenced with MUSCLE) and finally, nucleotides were reinserted in place of the amino acids. The four PCR primers (Ahne 5-8) were then positioned into their binding sites to assess their similarity to the corresponding area of the Sunshine virus genome. * = complementary sequence.
**Sunshine virus** (3': 2146-2177)  
ATAGAGGGGATTACAAAAAGAATTGGTCAGC  
**PAR-F1** (5' → 3')  
GAAGTATTGTCAIARNTNRTGGAC  
**FDLV** (3': 2200-2231)  
ATTGAGGGGATTACAAAAAGAATTGGTCATAT  
**Sunshine virus** (3': 2224-2254)  
ATTGCGGCTTCTATTGTCAAGGAGATAACCA  
**PAR-F2** (5' → 3')  
GTTGTATATTGTTCARGGGGAYAA  
**FDLV** (3': 2278-2309)  
AGAGGTTCGAGTTATCAAGGAAGACAAATA  
**Sunshine virus** (3': 2781-2812)  
CCAACGATTGATGATGATGATGATTCCAGAGTA  
**PAR-R** (3' → 5')  
GNAAYATHGIGAIICIGTAATTCCAGC  
**FDLV** (3': 2835-2866)  
GTTAATATCGGGATCTTTAGTACCGGAT  
**Sunshine virus** (3': 1695-1726)  
ACACGTGTAGATGATTTGAAAAATGACCTT  
**PNE-F1** (5' → 3')  
GTGTAAGTATGTGYGNNATGACGCC  
**Bovine RSV** (3': 1860-1891)  
TAGTGTAAGTATGGATTGCCAAGCAAGGAC  
**Sunshine virus** (3': 1912-1943)  
ATTACGTATTGTTAAATGGATACTGTPTAAGATG  
**PNE-F2** (5' → 3')  
ACTGATTIAAYAARTTTTAAAYCAGQ  
**Bovine RSV** (3': 2083-2114)  
ATTACGTATTGTTAAATGGATAATCAGGTATTT  
**Sunshine virus** (3': 2147-2177)  
TAGAGGGGATTACAAAAAGAATTGGTCAGC  
**PNE-R** (3' → 5')  
GARGGNTGTTGYCAAAATTGTTGGGAC  
**Bovine RSV** (3': 2321-2351)  
TAGAGGGGATTACAAAAATGGGTGGCATA

**Figure 6.9** Assessment of the binding affinity of the Tong L-gene PCR primer set (Tong et al., 2008) to Sunshine virus, Fer-de-Lance virus (FDLV) and Bovine respiratory syncytial virus (RSV). A translational alignment was performed using the same method described in **Figure 6.8.** The six PCR primers (PAR-F1, PAR-F2, PAR-R, PNE-F1, PNE-F2, PNE-R) were then positioned into their binding sites to assess their similarity to the corresponding area of the Sunshine virus genome. * = complementary sequence.
Ahne 5 (20)  
FDLV, 19 (95)  
Sunshine virus, 6 (30)  
FDLV, √√√√√√
Sunshine virus, x√xx√

Ahne 6 (21)  
FDLV, 21 (100)  
Sunshine virus, 13 (62)  
FDLV, √√√√√√
Sunshine virus, x√xx√

Ahne 7 (19)  
FDLV, 19 (100)  
Sunshine virus, 7 (37)  
FDLV, √√√√√√
Sunshine virus, x√xx√

Ahne 8 (21)  
FDLV, 21 (100)  
Sunshine virus, 15 (71)  
FDLV, √√√√√√
Sunshine virus, x√xx√

PAR-F1 (26)  
FDLV, 22 (85)  
Sunshine virus, 20 (77)  
FDLV, √√√√√√
Sunshine virus, √xx√x

PAR-F2 (26)  
FDLV, 18 (69)  
Sunshine virus, 21 (81)  
FDLV, √√√√√√
Sunshine virus, √xx√x√

PAR-R (28)  
FDLV, 22 (79)  
Sunshine virus, 17 (61)  
FDLV, √√√√√√
Sunshine virus, √xx√x√

PNE-F1 (28)  
Bovine RSV, 28 (100)  
Sunshine virus, 15 (54)  
Bovine RSV, √√√√√√
Sunshine virus, xx√xx√

PNE-F2 (26)  
Bovine RSV, 26 (100)  
Sunshine virus, 16 (62)  
Bovine RSV, √√√√√√
Sunshine virus, xx√xx√

PNE-R (27)  
Bovine RSV, 24 (89)  
Sunshine virus, 16 (59)  
Bovine RSV, √√√√√√
Sunshine virus, xx√xx√

| Table 6.3 Comparison of the primer binding sites on either Fer-de-Lance virus (FDLV) or bovine respiratory syncytial virus (RSV) compared to Sunshine virus for each of ten primers. The number of bases that are identical to the primer sequence are presented, as are the similarities that exist at the 3’ end of the primer. |
| --- | --- | --- |
| Primer (bases) | Virus, Primer matches (%) | Virus, Primer matches (√) or mismatches (x) for the six bases at the 3’ end of the primer (5’ → 3’) |
| Ahne 5 (20) | FDLV, 19 (95) | FDLV, √√√√√√ |
| Sunshine virus, 6 (30) | Sunshine virus, x√xx√ |
| Ahne 6 (21) | FDLV, 21 (100) | FDLV, √√√√√√ |
| Sunshine virus, 13 (62) | Sunshine virus, x√xx√ |
| Ahne 7 (19) | FDLV, 19 (100) | FDLV, √√√√√√ |
| Sunshine virus, 7 (37) | Sunshine virus, x√xx√ |
| Ahne 8 (21) | FDLV, 21 (100) | FDLV, √√√√√√ |
| Sunshine virus, 15 (71) | Sunshine virus, x√xx√ |
| PAR-F1 (26) | FDLV, 22 (85) | FDLV, √√√√√√ |
| Sunshine virus, 20 (77) | Sunshine virus, √xx√x |
| PAR-F2 (26) | FDLV, 18 (69) | FDLV, √√√√√√ |
| Sunshine virus, 21 (81) | Sunshine virus, √xx√x√ |
| PAR-R (28) | FDLV, 22 (79) | FDLV, √√√√√√ |
| Sunshine virus, 17 (61) | Sunshine virus, √xx√x√ |
| PNE-F1 (28) | Bovine RSV, 28 (100) | Bovine RSV, √√√√√√ |
| Sunshine virus, 15 (54) | Sunshine virus, xx√xx√ |
| PNE-F2 (26) | Bovine RSV, 26 (100) | Bovine RSV, √√√√√√ |
| Sunshine virus, 16 (62) | Sunshine virus, xx√xx√ |
| PNE-R (27) | Bovine RSV, 24 (89) | Bovine RSV, √√√√√√ |
| Sunshine virus, 16 (59) | Sunshine virus, xx√xx√ |

From this data, it can be seen that there was near perfect similarity between all four Ahne L gene primers and FDLV but for Sunshine virus, there are a large number of mismatches. Only Ahne 6 has five consecutive matches at its 3’ end with Sunshine virus. Similarly, with the Tong primers, only PAR-F2 and PNE-R showed strong 3’ end similarities with Sunshine virus. Ahne 6 (a reverse primer) is upstream from PAR-F2 (a forward primer) and so would not generate an amplicon. Equally, PNE-R (a reverse primer) is also upstream from PAR-F2 and so this primer pair would not produce an amplicon either.

By analysing the primer binding sites of various paramyxoviral primers to Sunshine virus using these methods, it could be seen that they would not be anticipated to amplify a segment of the viral genome. Even with the few primers that would be assumed to bind (Ahne 6, PAR-F2 and PNE-R), they cannot be used in concert due to their respective binding sites. However, this
A method of primer binding analysis could prove useful in designing primers or using existing primers (from different published sets) to be able amplify Sunshine virus. It would be even more advantageous if primers could be designed that could amplify both ferlaviruses and Sunshine virus in a way that produced amplicons that were different sizes for each virus. This would allow both viruses to be screened with only one primer set. The divergent sequences of these two viruses could allow this possibility.

Although this analysis had shown potential in identifying primers that could amplify Sunshine virus, it was ultimately shown that it was unlikely that technical reasons (e.g. number of cycles, annealing temperature, concentration of magnesium chloride, primer concentration, amount of starting template etc) explained the inability of these primers to amplify a segment of Sunshine virus. It was therefore concluded that new primers should be designed for the detection of Sunshine virus.

Sunshine virus had been discovered as a novel paramyxovirus but several questions still remained about its significance.

1. Could this virus be detected in uninfected VH2 cells?
2. Could this virus be detected in BHP1-Lung (the tissue sample)?
3. Could this virus be detected in the tissue samples of other snakes that produced the same cytopathic effects (CPE) in cell culture?
4. Could this virus be detected in the tissues of seemingly healthy snakes?
5. Could this virus be detected in formalin-fixed paraffin-embedded (FFPE) samples?

A set of PCR primers that was specifically designed for Sunshine virus would provide a rapid, sensitive and specific means to detect this virus in a range of samples, allowing many of these questions to be answered. Using the paramyxoviral sequence information that had been
generated from high-throughput sequencing, non-degenerate primers were designed based on the RNA-dependent RNA-polymerase gene. This gene was selected as it has been shown to be one of the most conserved regions between different paramyxoviruses (Kurath et al., 2004), and, for snake paramyxoviruses, between different ferlaviruses (Marschang et al., 2009). Targeting the polymerase gene would hopefully facilitate the future detection of other strains of Sunshine virus. 100 primer sequences were produced by a computer program (Primer Premier®, Version 5, PREMIER Biosoft International) and from these, four primers were chosen (Table 6.4) for several reasons: similar melting temperatures; minimal predicted hairpin, dimer and cross-dimer formation; weak annealing to other areas of the Sunshine virus genome (false-binding); BLASTN (Basic Local Alignment Search Tool for Nucleotides) failed to find similar sequences in the GenBank database; the ability to be used in different combinations (Figure 6.10); and at least some of these primers would produce amplicons small enough to detect Sunshine virus in wax-embedded archival material.

<table>
<thead>
<tr>
<th>Primer set (number of nucleotides from 3’ end of polymerase gene)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SunshineS1 (2444): 5’GGAAAGGGAGGTCTATG</td>
<td>153</td>
</tr>
<tr>
<td>SunshineAS1 (2596): 5’ATTCAACATCTGGGGTC</td>
<td></td>
</tr>
<tr>
<td>SunshineS2 (2240): 5’TTCAAGGAGATAACCAGG</td>
<td>230</td>
</tr>
<tr>
<td>SunshineAS2 (2469): 5’CGGGATTCCCATAGAC</td>
<td></td>
</tr>
<tr>
<td>SunshineS1 (2596): 5’ATTCAACATCTGGGGTC</td>
<td>357</td>
</tr>
</tbody>
</table>

Table 6.4 Primer sequences, and anticipated amplicon size, for the detection of Sunshine virus. PCR = polymerase chain reaction. bp = base pairs.
Figure 6.10 Four primers designed for the detection of Sunshine virus: S1, S2, AS1 and AS2. Each amplicon (represented as rectangles) starts and finishes with the number of bases from the 3’ end of the polymerase gene. Primers can be used as three separate primer pairs (S1-AS1 = 153bp product; S2-AS2 = 230bp product; S2-AS1 = 357bp product) or in two heminested polymerase chain reactions (S2-AS1 followed by S1-AS1; or S2-AS1 followed by S2-AS2). bp = base pairs.

These primers were then used with various templates to answer the five questions mentioned earlier.

Experiment one – infected versus uninfected viper heart cells

Detecting this paramyxovirus in infected but not uninfected VH2 cells would suggest that this virus was from the tissue sample (BHP1-Lung) that was inoculated onto the VH2 cells and was not a contaminant or a virus endogenous to this cell line. To investigate this, attempts were made to detect Sunshine virus in uninfected cells using all three Sunshine virus primer pairs (AS1-S1, AS2-S2 and AS1-S2). Infected cells served as positive controls.

RNA was extracted and cDNA was synthesised from viper heart cells according to the methods described earlier (section 4.8 – Polymerase Chain Reaction) All Sunshine virus PCRs used the same cycling conditions: 94°C x 2 minutes, followed by 40 cycles of 94°C x 20 seconds, 45°C x 45 seconds and 72°C x 30 seconds. Sunshine virus could be detected in two flasks of viper heart cells that had been infected with BHP1-Lung and JCP1NT-Brain (abbreviations defined in
Table 5.3) respectively (Figure 6.11). All three primer pairs yielded an amplicon of the appropriate size in infected cells and did not produce any amplicons using the RNA from uninfected cells as a template. DNA was extracted from agarose gel and sequenced directly as previously described (section 4.8 – Polymerase Chain Reaction). Sequenced PCR products were compared to the relevant section of the Sunshine virus genome that had been determined by high-throughput sequencing.

![Polymerase chain reaction amplicons of uninfected and infected viper heart cells. Infected cells had either been inoculated with BHP1-Lung or JCP1NT-Brain. Lanes 1-3 = primer pair AS1-S1 (153bp); lane 1 = uninfected cells, lane 2 = BHP1-Lung, lane 3 = JCP1NT-Brain. Lanes 4-6 = primer pair AS2-S2 (230bp); lane 4 = BHP1-Lung, lane 5 = JCP1NT-Brain, lane 6 = uninfected cells, Lane 7 = molecular ladder. 100bp increments from 100-1,000bp, brightest band is 500bp, largest band is 1,500bp. Lanes 8-10 = primer pair AS1-S2 (357bp); lane 8 = BHP1-Lung, lane 9 = JCP1NT-Brain, lane 10 = uninfected cells.]

**Figure 6.11**

*Experiment two – infected versus uninfected tissue samples*

Detecting Sunshine virus in viper heart cells infected with BHP1-Lung and JCP1NT-Brain, and not in uninfected viper heart cells, suggested that Sunshine virus was not endogenous to this cell line and the origin of the virus was from the tissue samples that been inoculated onto these cells. However, it was still conceivable that Sunshine virus was a contaminant that infected some (but not all) VH2 cells during virus isolation attempts. To explore this possibility, the original tissue sample, BHP1-Lung, was tested for Sunshine virus by PCR. In addition, a sample of BHP1-Lung that had previously been formalin-fixed and paraffin-embedded (FFPE) was used to see if Sunshine virus (if present) could be detected in archival samples. To serve as a
negative control, “unaffected” snake tissue was used. The pathology archives of Murdoch University were searched for snake submissions where the cause of death had been attributed to a non-viral aetiology (e.g. road trauma or neoplasia). RNA was extracted from FFPE and fresh-frozen tissue samples according to the methods described earlier (section 4.8 – Polymerase Chain Reaction). For reverse transcription, cDNA was synthesised using random hexamers or gene-specific primers (GSP). cDNA synthesis using random hexamers was performed as previously described (section 4.8 – Polymerase Chain Reaction). For gene-specific primers, the same method was employed but instead of 1µL of random hexamers (100ng/µL), 0.5µL of each primer (~1µM final concentration) was used.

For PCR, 10µL of SYBR GreenER™ SuperMix (Invitrogen, Victoria, cat. no. 11762-100) was combined with each primer (AS1-S1, 153bp amplicon) to a final concentration of 1µM and made up to a final volume of 19µL using PCR-grade water. To this, 1µL of cDNA was added. The PCR was performed in a Rotor-Gene thermocycler (RG-3000, Corbett Research, Sydney, Australia) using cycling conditions previously described for this primer pair. Sunshine virus could be detected in both the freshly-frozen and FFPE samples of BHP1-Lung, but not in the tissue of a snake that was unlikely to be infected with Sunshine virus (6.12). Virus was detected irrespective of the method of cDNA synthesis but when synthesised with gene-specific primers, the cycle threshold (Ct value) was approximately 1-2 cycles lower than cDNA that had been amplified using random hexamers (data not shown). The linear range of quantitation of this real-time PCR had not been established and so these results should not be over-interpreted.
Figure 6.12 Polymerase chain reaction of tissue samples of BHP1-Lung. Lanes 1-7 = primer pair AS1-S1 (153bp); lane 1 = positive control (viper heart cells infected with BHP1-Lung), lane 2 = tissue sample BHP1-Lung – random hexamers, lane 3 = tissue sample BHP1-Lung – gene-specific primers (GSP), lane 4 = formalin-fixed paraffin-embedded (FFPE) BHP1-Lung – random hexamers, lane 5 = FFPE BHP1-Lung – GSP, lane 6 = FFPE negative control (“unaffected” snake tissue) – random hexamers, lane 7 = FFPE negative control – GSP, lane 8 = molecular ladder. 100bp increments from 100-1,000bp, brightest band is 500bp, largest band is 1,500bp.

From these two simple experiments, numerous conclusions could be drawn:

• Sunshine virus does not appear to be a virus that is endogenous to the viper heart cell line,

• Sunshine virus was only found in tissue samples that produced cytopathic effects in cell culture consistent with a paramyxovirus,

• Sunshine virus could not be found in the tissues of a snake unlikely to have been infected with Sunshine virus, and

• Using the methods described, Sunshine virus could be detected in FFPE material.

With these preliminary experiments completed, these primers could be applied to a range of samples to further investigate the link between Sunshine virus and disease.

6.6 Chapter Summary

This chapter describes the molecular identification of BHP1-Lung as a novel paramyxovirus that was then named Sunshine virus. High-throughput sequencing was performed on RNA from viper heart cells infected with BHP1-Lung and more than 10 million reads were sequenced
(each 38 nucleotides long). From these, 5,818 unique contigs were assembled but only one was confirmed to be viral in origin. The translated sequence of this contig contained open reading frames (ORF) that showed strong similarity to the nucleoprotein (N), matrix (M), fusion (F) and polymerase (L) proteins found in paramyxoviruses. BLASTN and BLASTP (Basic Local Alignment Search Tool for Nucleotides [BLASTN] / Proteins [BLASTP]) searches could not find any similarities in the GenBank database to the putative attachment protein or the putative phosphoprotein of BHP1-Lung. In the case of the putative attachment protein, it showed a similarity of less than 15% to 18 other paramyxoviral attachment proteins. All these results confirmed BHP1-Lung as a novel paramyxovirus and it was named Sunshine virus, after the geographical origin of the first isolate: the Sunshine Coast of Queensland, Australia. Work performed by Dr Jim Wellehan phylogenetically placed Sunshine virus within the family Paramyxoviridae but outside of both existing subfamilies.

Two paramyxovirus primer sets (Ahne et al., 1999b; Tong et al., 2008) were analysed for their appropriateness to detect Sunshine virus. The results of this analysis supported the experimental work performed in the previous chapter that had suggested that these primers may be unsuitable for the detection of Sunshine virus. Because of this, new primers were designed that were able to demonstrate that Sunshine virus: (1) was not endogenous to the viper heart cell line, (2) could be detected in the BHP1-Lung tissue sample and, (3) could not be found in “unaffected” snake tissue. These primers were also able to detect Sunshine virus in formalin-fixed paraffin-embedded (FFPE) archival material.

In the next chapter, the clinical signs and pathological findings that are associated with Sunshine virus are discussed. The results of PCR testing on a number of samples are also reported.
Chapter 7 – Diagnosing Sunshine virus

In chapter three (Paramyxoviruses in Australian Snakes), background information on an ongoing problem in Australian snakes was outlined. Snakes were dying with neurorespiratory signs of disease, and pathological findings and overseas testing indicated that at least some of these snakes were likely to have been infected with one, or a combination of, a small selection of pathogenic viruses. A paramyxovirus provided the soundest explanation for these initial findings but a reovirus or a retrovirus could not be excluded either. The purpose of the present project was to search for these viruses in Australian snakes with neurorespiratory clinical signs.

After analysing 463 snake samples, using a variety of methods, a paramyxovirus was discovered (Chapter 5 – Sample Examination Leading to the Isolation of a Virus Named BHP1-Lung). Significantly, this paramyxovirus was the first to be isolated from a reptile that did not belong to the proposed genus Ferlavirus (Chapter 6 – Molecular Identification of BHP1-Lung as a Novel Paramyxovirus Named Sunshine virus). This virus was named Sunshine virus after the geographical origin of the first isolate: the Sunshine Coast of Queensland, Australia. It was determined that existing paramyxovirus primers would probably be inadequate to screen samples for Sunshine virus by polymerase chain reaction (PCR). New primers were designed and promising test results on positive control material provided a justification to use these primers on clinical samples.

The first section of this chapter (7.1 – Results of Polymerase Chain Reaction Testing for Sunshine virus) describes the PCR testing for Sunshine virus that was performed on a range of clinical samples (swabs, tissues and formalin-fixed paraffin-embedded tissues). PCR results that were positive provided a means to identify snakes that were infected with Sunshine virus. This then allowed the clinical signs and pathological findings that were associated with Sunshine virus infection to be reported. This clinical data is presented in the next three sections (7.2 –
Clinical Signs, 7.3 – Gross Pathology, and 7.4 – Histopathology). The final section summarises the findings presented in this chapter (7.5 – Chapter Summary).

7.1 Results of Polymerase Chain Reaction Testing for Sunshine virus

From October-2010 to August-2011, 141 samples from three separate submissions were tested for Sunshine virus by PCR (Table 7.1). The first two sample submissions were described earlier (section 5.2.7 – Virus Isolation) but will also be mentioned here briefly. The third submission is presented below for the first time.

Sample Submission One – an Outbreak of Neurorespiratory Disease in a Private Collection from the Sunshine Coast of Queensland

In 2008, all the snakes (70 Australian pythons) in a private collection were humanely euthanased in response to an outbreak of neurorespiratory disease. A more detailed history of this outbreak may be found starting on page 152. In total, samples from 17 livers, kidneys and lungs, 16 brains and 13 serum samples were collected from 17 snakes. Snakes were selected for sample collection based on clinical signs and/or which snakes they had been in direct contact with. Half of each organ sample was submitted frozen and the other half was submitted fixed in formalin.

Five viruses were isolated from frozen tissue samples (Table 5.3) and one of these, a lung sample from a black-headed python (BHP1-Lung), was identified as Sunshine virus using high-throughput sequencing and a set of newly-designed PCR primers

9 (Figures 6.11 and 6.12). Besides BHP1-Lung, 79 frozen samples (samples from 17 livers and kidneys, 16 lungs and brains and 13 serum samples) from this collection were tested for the presence of Sunshine virus by PCR.

9 Of the five viruses isolated from Sample Submission One and the four viruses isolated from Sample Submission Two, only two were identified as Sunshine virus by PCR and sequencing: BHP1-Lung and JCP1NT-brain. However, all the tissue samples that were used to isolate these viruses, were tested by PCR and sequencing to confirm the presence of Sunshine virus.
Sample Submission Two – Samples collected by Dr Cathy Shilton from the Berrimah Veterinary Laboratories (BVL) in Darwin, Northern Territory

From 2005 to 2008, Dr Cathy Shilton, an anatomical veterinary pathologist from the Northern Territory (NT), identified nine snakes from four separate NT locations that had histories, clinical signs and/or pathological findings that were considered consistent with a viral (potentially paramyxoviral) infection. Formalin-fixed paraffin-embedded (FFPE) material was available from all nine snakes. Additionally, freshly-frozen samples from three brains and two lungs were available from four of these snakes.

Four viruses were isolated using viper heart cells (Table 5.3) and one of these, JCP1\textsubscript{NT}-Brain, was later confirmed to be Sunshine virus by PCR\textsuperscript{9} (Figure 6.11). Only formalin-fixed paraffin-embedded samples were tested for the presence of Sunshine virus using PCR.

Sample Three – An Outbreak of Neurological Disease in a Queensland Zoological Collection\textsuperscript{10}

In 2011, a collection of 32 snakes (24 Australian and exotic non-venomous snakes and eight venomous snakes) was afflicted by sporadic cases of neurological disease and other non-specific signs of disease (inappetence, lethargy and regurgitation). Cloacal and oral swabs were opportunistically sampled on multiple occasions. From some of these animals, three swabs were collected: an oral-only swab, a cloacal-only swab and a combined oral-cloacal swab. Methods for swabbing were described earlier (section 4.1 – Sample Collection). In total, 41 combined oral-cloacal swabs, three oral-only and three cloacal-only swabs from 22 snakes were tested for Sunshine virus by PCR. In addition, a sample of brain, lung, liver and kidney from one of these snakes was also tested.

\textsuperscript{10} It should be noted that this is the sixth zoological collection mentioned in this thesis. In Chapter 3, four Australian zoological collections were mentioned that had pursued testing for anti-ferlaviral antibodies at an overseas laboratory. The Queensland zoological collection mentioned in Chapter 5 that produced the haemagglutinating samples (samples 3 and 7) was one of these four Australian zoological collections. The final mention of a zoological collection, described in this chapter for the first time, is distinct from all others previously mentioned. These collections have been ambiguously named to retain their anonymity.
### Sampling Data

<table>
<thead>
<tr>
<th></th>
<th>Sample Submission One</th>
<th>Sample Submission Two</th>
<th>Sample Submission Three</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Private Collection, Sunshine Coast, Queensland</td>
<td>Berrimah Veterinary Laboratories (BVL), Northern Territory</td>
<td>Queensland Zoological Collection</td>
</tr>
<tr>
<td>Period of Sampling</td>
<td>2008</td>
<td>2005 to 2008</td>
<td>2011</td>
</tr>
<tr>
<td>Collections Sampled</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Snakes Sampled</td>
<td>17</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>Virus isolation results</td>
<td>Five isolates from two snakes, one confirmed to be Sunshine virus</td>
<td>Four isolates from two snakes, one confirmed to be Sunshine virus</td>
<td>No previous testing</td>
</tr>
</tbody>
</table>

### Samples

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Submission One</th>
<th>Sample Submission Two</th>
<th>Sample Submission Three</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>16</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Lung</td>
<td>17</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Liver</td>
<td>17</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Kidney</td>
<td>17</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Various formalin-fixed paraffin-embedded tissues</td>
<td>17</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Serum</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Combined oral-cloacal swabs</td>
<td>0</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Cloacal-only swabs</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Oral-only swabs</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total Samples Tested by PCR</strong></td>
<td><strong>81</strong>*</td>
<td><strong>9</strong>*</td>
<td><strong>51</strong>*</td>
</tr>
</tbody>
</table>

*Only one formalin-fixed paraffin-embedded (FFPE) sample was tested (Figure 6.12), the remaining 80 samples consisted of freshly frozen tissue samples and serum. #Only FFPE material was tested by PCR.*
RNA was extracted and then reverse transcribed into cDNA with random hexamers, from swabs, freshly-frozen tissue samples and FFPE material as previously described (section 4.8 – Polymerase Chain Reaction). The primer set SunshineS1-SunshineAS1 (153bp amplicon, Table 6.4) was always used for FFPE material but for other samples, SunshineS2-SunshineAS1 (357bp amplicon, Table 6.4) was usually used. Cycling conditions for the PCR were described earlier (section 6.5 – Development and Validation of a Sunshine virus Polymerase Chain Reaction Primer Set, Experiment one, page 227). Sequencing of PCR products was in accordance with previously described methods (section 4.8 – Polymerase Chain Reaction). For all snakes that were PCR tested for Sunshine virus using swab samples, the combined oral-cloacal swab was tested first. If a snake tested positive, and individual swabs were available, the individual swabs were then tested to determine whether the oral-only and/or the cloacal-only swabs were positive.

7.1.2 PCR Testing on Tissue Samples and Serum, Including a Comparison to Virus Isolation Results

Results of PCR testing on tissue samples were compared to the relevant virus isolation results. Positive results are presented in Table 7.2.
<table>
<thead>
<tr>
<th>Species</th>
<th>Sample Submission</th>
<th>Clinical History</th>
<th>Polymerase Chain Reaction (PCR)</th>
<th>Virus Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brain</td>
<td>Lung</td>
</tr>
<tr>
<td>Jungle carpet python 1</td>
<td>1</td>
<td>Clinical respiratory disease</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Jungle carpet python 2</td>
<td>1</td>
<td>Slight head tremor</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Jungle carpet python 3</td>
<td>1</td>
<td>Slight head tremor</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Jungle carpet python 4</td>
<td>1</td>
<td>Subtle neurological signs. Shared enclosure with snake that died acutely</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Black-headed python 1</td>
<td>1</td>
<td>Low grade respiratory disease and stomatitis. Shared enclosure with two snakes (one after the other) that died acutely</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Black-headed python 2</td>
<td>1</td>
<td>Asymptomatic. Mated to snake that later died acutely</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Woma python</td>
<td>1</td>
<td>Chronic respiratory signs and periods of neurological dullness</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spotted python 2</td>
<td>3</td>
<td>Weakness and lack of coordination</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Total positive / total samples tested**

8/8 5/8 5/8 7/8 0/6

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jungle carpet python 1</td>
<td>+</td>
</tr>
<tr>
<td>Jungle carpet python 2</td>
<td>-</td>
</tr>
<tr>
<td>Jungle carpet python 3</td>
<td>+</td>
</tr>
<tr>
<td>Carpet python 1</td>
<td>+</td>
</tr>
<tr>
<td>Carpet python 2</td>
<td>+</td>
</tr>
<tr>
<td>Carpet python 3</td>
<td>+</td>
</tr>
</tbody>
</table>

**Total positive / total samples tested**

4/10 3/10 2/7

Table 7.2 PCR and virus isolation results from snakes that were positive for Sunshine virus by either test. Positive virus isolation was defined by a characteristic cytopathic effect (large syncytium with minimal cell lysis). - = negative. + = positive. n.s. = not sampled. n.t. = not tested. Ki-Li = combined kidney-liver sample. BVL = Berrimah Veterinary Laboratory. 2A/2B represents separate collections. *PCR performed on pooled formalin-fixed paraffin-embedded (FFPE) organs that included at least brain and lung. All individual organ samples were submitted fresh or freshly-frozen. Jungle carpet python = *Morelia spilota cheynei*. Black-headed python = *Apidites melanocephalus*. Woma python = *Aspidites ramsayi*. Spotted python = *Antaresia maculosa*. Carpet python = *Morelia spilota*. The “Spotted python 2” in this table is the same snake as the “Spotted python 2” mentioned in Table 7.3.
The results in Table 7.2 show that Sunshine virus has been detected in fresh-frozen tissues and formalin-fixed paraffin-embedded (FFPE) sections from a range of Australian pythons.

In this investigation, there were eight snakes that were PCR positive in at least one of the four freshly-frozen organs. Seven snakes were PCR positive in more than one of organs sampled. A sample of brain was PCR positive in all eight cases (8/8). Samples of kidney (7/8), liver (5/8) and lung (5/8) were also commonly positive by PCR. The data presented here are exploratory and are limited by a small number of samples that were PCR positive but it would seem reasonable that sampling the brain during a necropsy should form a priority sample during an investigation of suspected Sunshine virus infection. Similarly, taking a sample of (in order of preference) kidney, liver and lung from a live animal may be useful when screening for this virus.

Within this group of eight snakes, all snakes had been identified to be infected with Sunshine virus by PCR but Sunshine virus was isolated in only two of these animals. Furthermore, more freshly-frozen organ samples were positive by PCR (25/32 = 78%) than virus isolation (5/21 = 24%). No sample was positive by virus isolation and negative by PCR. To standardise this comparison further, spotted python 2 can be excluded from this group of eight snakes because it was not tested by virus isolation and PCR, and the PCR testing on kidney and liver samples can be combined (if either kidney or liver was positive by PCR, the “combined” sample is considered positive). After this data standardisation, PCR was still positive (17/21 = 81%) more often than virus isolation (5/21 = 24%).

Two other papers have compared PCR to virus isolation in detecting reptilian paramyxoviruses. Both papers refer to the ferlaviruses. In a study by Kolesnikovas et al. (2006), using organ samples from three snakes infected with ferlavirus, both virus isolation (six positives out of seven samples) and PCR (three positives out of three samples) were able to detect infection in
all three snakes. In a second study with a much larger data set, 203 samples (organs, swabs and tracheal washes) from 102 snakes were tested for the presence of ferlaviruses by PCR and virus isolation (Papp et al., 2010a). From these 102 snakes, at least one organ sample was either PCR- or virus isolation-positive in 16 snakes (in our data set, fresh organ samples from seven snakes, that were tested by virus isolation and PCR, were positive by PCR or virus isolation). Of these 16 snakes tested by Papp et al. (2010a), 36 out of 51 organ samples were PCR-positive (71%) and three were positive by virus isolation (3/51 = 6%). Of the seven snakes from our data set, 17 out of 21 organ samples (pooling the results of liver and kidney) were positive by PCR (81%) and five were positive by virus isolation (5/21 = 24%). There are several reasons why our PCR and virus isolation results cannot be directly compared to those of Papp et al. (2010a). For example, different paramyxoviruses were of interest, the organs that were sampled were not standardised and the PCR and virus isolation methods were not the same. However, in both investigations, PCR detected the paramyxovirus of interest more reliably than virus isolation.

The samples from the second submission, Berrimah Veterinary Laboratories (BVL), were tested by virus isolation and PCR (Table 7.2). Freshly-frozen samples were used for virus isolation and FFPE material was used for PCR. Under these testing conditions, Sunshine virus was detected more commonly by PCR (7/8 = 88%) than virus isolation (4/6 = 67%). In one snake from this submission, jungle carpet python 2, a virus was isolated but PCR testing on FFPE material was negative. This snake was part of a collection (2A) where a virus had been isolated in other snakes that had been identified as Sunshine virus by PCR, so the virus isolated from jungle carpet python 2 was likely the same virus suggesting that the PCR result was a false negative. Sample degradation in the FFPE material would provide one explanation for this result.

No serum sample tested positive for Sunshine virus by PCR (n=13) but considering the onset and duration of viraemia has not been determined, the significance of this finding is undefined.
7.1.3 PCR Testing on Swab Samples

Swab samples were collected from Sample Submission Three over a 105-day period and tested for Sunshine virus by PCR. Results are summarised in Table 7.3.
<table>
<thead>
<tr>
<th>Species</th>
<th>Clinical History</th>
<th>day 0</th>
<th>day 19</th>
<th>day 45</th>
<th>day 105</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spotted python 1 (<em>Antaresia maculosa</em>)</td>
<td>Asymptomatic</td>
<td>-</td>
<td>n.s.</td>
<td>deceased(^{a})</td>
<td>n/a</td>
</tr>
<tr>
<td>Diamond python (<em>Morelia spilota spilota</em>)</td>
<td>Weakness, regurgitation, decreased righting reflex</td>
<td>-</td>
<td>+</td>
<td>deceased(^{a})</td>
<td>n/a</td>
</tr>
<tr>
<td>Spotted python 2</td>
<td>Weakness, lack of coordination</td>
<td>n.s.</td>
<td>-</td>
<td>euthanased(^{b})</td>
<td>n/a</td>
</tr>
<tr>
<td>Spotted python 3</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spotted python 4</td>
<td>Weakness</td>
<td>n.s.</td>
<td>n.s.</td>
<td>+(^{a})</td>
<td></td>
</tr>
<tr>
<td>Spotted python 5</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td>+(^{a})</td>
<td></td>
</tr>
<tr>
<td>Coastal carpet python 1 (<em>Morelia spilota mcdowelli</em>)</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coastal carpet python 2</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coastal carpet python 3</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coastal carpet python 4</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coastal carpet python 5</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td>n.s.</td>
</tr>
<tr>
<td>Coastal carpet python 6</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coastal carpet python 7</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coastal carpet python 8</td>
<td>Weakness, lethargy, inappetence</td>
<td>n.s.</td>
<td>n.s.</td>
<td>+(^{a})</td>
<td>n.s.</td>
</tr>
<tr>
<td>Coastal carpet python 9</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Coastal carpet python 10</td>
<td>Asymptomatic</td>
<td>n.s.</td>
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</tr>
<tr>
<td>Boa constrictor 1 (<em>Boa constrictor</em>)</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Boa constrictor 2</td>
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<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Boa constrictor 3</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Centralian carpet python (<em>Morelia spilota bredli</em>)</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
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</tr>
<tr>
<td>Jungle carpet python (<em>Morelia spilota cheynei</em>)</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
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</tr>
<tr>
<td>Scrub python (<em>Morelia amethistina</em>)</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
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</tr>
<tr>
<td>Yellow anaconda (<em>Eunectes notaeus</em>)</td>
<td>Asymptomatic</td>
<td>n.s.</td>
<td>n.s.</td>
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</table>

Table 7.3 Polymerase chain reaction (PCR) results of combined oral-cloacal swabs from a zoological collection in Queensland (Sample Submission Three). - = negative. + = positive. n.s. = not sampled. * = dead prior to this sampling date. a = organ samples not retrieved for further testing. b = organ samples retrieved for further testing. # = separate cloacal-only and oral-only swabs were also PCR positive. n/a = not applicable. The “Spotted python 2” in this table is the same snake as the “Spotted python 2” mentioned in Table 7.2.
During an outbreak of disease in a Queensland zoological collection, seven out of 32 snakes died and/or were positive for Sunshine virus by PCR (Table 7.3). Spotted python 1 was asymptomatic, tested negative on day zero and then died without further investigation. Whether this snake was infected with Sunshine virus is unknown.

The diamond python was symptomatic, tested negative on day zero, positive on day 19 and then died. Although this is the only example where a subsequent PCR test detected Sunshine virus where the first did not, this one result does suggest that serial sampling of animals may be important if trying to screen animals for the presence of Sunshine virus infection. This result does not provide enough data to recommend an appropriate sampling interval.

Spotted python 2 was symptomatic, tested negative on day 19, was later euthanased and organ samples were collected that tested positive. This case suggests that an organ sample is more likely to detect Sunshine virus than a combined cloacal-oral swab but there is insufficient data to validate this conclusion.

Spotted python 3 was asymptomatic, tested positive on day 19 but was then negative on days 45 and 105. Spotted pythons 4 (symptomatic) and 5 (asymptomatic) tested positive on all three swabs (oral-only, cloacal-only and combined oral-cloacal) on day 45 but combined oral-cloacal swabs collected on day 105 tested negative. Coastal carpet python 8 was symptomatic, tested positive on all three swabs on day 45 but was not tested at any other time. Although it may be tempting to think that this data may be able to provide an insight into the duration of Sunshine virus shedding, crucial pieces of information are missing. First, the start of the shedding period cannot be determined in any of these cases. Second, the data is limited to only a few cases. Third, the sampling period and intervals between samples may not have been adequate to identify if Sunshine virus is shed intermittently. To answer many of these questions, a controlled transmission experiment would be needed and this should form a
priority of future studies on Sunshine virus. The shedding duration of another paramyxovirus of snakes, ferlavirus, may extend for several months (Lloyd and Flanagan, 1991) but for paramyxoviruses in other species, the shedding periods are usually brief (Lamb and Parks, 2007; Aldous et al., 2010; Dortmans et al., 2011; MacLachlan and Dubovi, 2011).

Despite their limitations, these results from spotted pythons 3-5 and coastal carpet python 8 do still provide some useful information. All four of these snakes were still alive one month beyond the last time of sampling, and two of these snakes never displayed overt signs of disease, so a positive PCR swab was not always associated with imminent death. The value of an animal should be measured against the value of a collection but at this time there is insufficient evidence to recommend that an individual animal that tests positive by PCR for Sunshine virus on a combined oral-cloacal swab should be immediately euthanased.

Where the combined oral-cloacal swab was positive for Sunshine virus by PCR and oral-only and cloacal-only swabs were available (n=3), both the oral-only and cloacal-only swabs also tested positively. Superficially, this suggests that the mouth, the cloaca or both sites combined, form equally appropriate areas to sample. However, this is limited by a small number of cases and future work may provide different conclusions. Future work into this area should consider utilising real time PCR to provide an estimate of the viral load that is shed from each site. This could help determine the relative value in sampling each site.

7.1.4 General Comments about PCR Testing
The clinical signs associated with Sunshine virus infection are discussed in greater detail later (section 7.2 – Clinical Signs) but a brief discussion that compares these clinical signs to the PCR results is presented here. There was little correlation between clinical signs (if any) and the organs that Sunshine virus was subsequently detected in by PCR (Table 7.2). However, in all cases, organs were only collected during necropsy and therefore may not be an accurate reflection of whether the organ had been infected previously. A negative PCR result in the
presence of clinical disease may reflect clearance of the viral nucleic acid from an organ(s) that has not recovered from injury. Conversely, a positive PCR result in the absence of clinical disease may reflect the presence of viral nucleic acid from an organ that is still able to meet the animal’s needs. In these instances, histopathological assessment of these organs may have provided further insight into the association between tissue lesions and a PCR result. In summary, the data presented here suggest that clinical signs should not dictate sample selection.

PCR was able to positively detect Sunshine virus in pythons more often than virus isolation and so PCR is recommended for screening samples that may contain Sunshine virus that is presumed to be closely related to previously detected strains of this virus. However, given that virus isolation does not rely on previously known sequence information, it continues to be an important tool of viral discovery.

Non-specific bands were occasionally seen when using the primer pair SunshineAS1-SunshineS1 (153bp amplicon). On one occasion, a 167bp product was amplified and sequenced which was most closely related to an endonuclease (GenBank accession number: XM_003224137). Also with this primer pair, unsequenced bands that were approximately 400 and 900 nucleotides were often seen with total nucleic acid (DNA and RNA) templates derived from organ samples that were negative for Sunshine virus. Non-specific bands have not been detected using the primer pairs SunshineAS2-SunshineS2 (230bp amplicon) or SunshineS2-SunshineAS1 (357bp amplicon). All amplicons from all primer pairs that were determined to be Sunshine virus by sequencing (n=24) showed very little variation. Single-base sequence variation was seen in two positions but both mutations were silent (Figure 7.1). No sequence variations were detected within a single collection.
Figure 7.1 Partial sequences of three Sunshine virus polymerase genes amplified using the primer pair, SunshineAS1 and SunshineS2. Each sequence is compared to black-headed python 1 (Collection 1) and where there is no sequence variation a period (.) is inserted into the corresponding nucleotide position. Two base positions are marked with an asterisk (*) where silent mutations have been detected. SS = Sample Submission. Refer to page 234 for descriptions of each sample submission.
In the Queensland zoological collection (Sample Submission Three) there were also eight venomous snakes from the black snake (*Pseudechis* spp.), taipan (*Oxyuranus* spp.), death adder (*Acanthophis* spp.) and tiger snake (*Notechis* spp.) genera. None of these snakes were showing overt signs of ill-health. For human safety reasons, these snakes were not sampled and so the susceptibility of these genera is unknown. Therefore, at this time, the range of host species that are susceptible to Sunshine virus cannot be reported beyond the family *Pythonidae*.

In conclusion, the data presented here make it difficult to make specific recommendations about optimum sample selection when screening snakes for infection with Sunshine virus. Therefore, collecting serial swabs and recovering samples of the brain, liver, kidney and lung for the purposes of PCR testing would seem to be the most appropriate recommendation that can be made at this time.

### 7.2 Clinical Signs

There were a variety of clinical signs in animals that were either PCR or virus isolation positive for Sunshine virus. Some infected animals displayed no overt signs of disease while for others, the clinical signs were neurological, neurorespiratory or non-specific. Neurological signs included head tremors, opisthotonus, incoordination, diminished righting reflexes, uncoordinated movement of the cranial and caudal body and erratic mouth gaping. Respiratory signs included a mild discharge of clear viscous fluid from the mouth and dyspnoea. Non-specific signs included anorexia, stomatitis, weakness, lethargy, regurgitation and inappetence. This suggests that the presence of neurorespiratory disease should alert the clinician’s mind to the possibility of Sunshine virus infection but also, the absence of it is non-excluding of this disease. These clinical signs should not be seen as pathognomonic for Sunshine virus as they would be difficult to differentiate from the signs associated with inclusion body disease (IBD) and infection with ferlavirus (*Table 2.1*) or reovirus (Girling and Raiti, 2004; Jacobson, 2007).
The pathological findings associated with Sunshine virus infection were reported by Dr Cathy Shilton (BSc, DVM, DVSc, MACVSc) from Berrimah Veterinary Laboratories (BVL). These findings contributed to a submitted manuscript about diagnosing Sunshine virus. Dr Shilton is an experienced and published anatomical veterinary pathologist. In these next two sections (7.3 – Gross Pathology and 7.4 – Histopathology) work is presented in text boxes that was not performed by the author of this thesis. It is included here nevertheless so that this thesis can contain the most complete account of the current knowledge on Sunshine virus.

### 7.3 Gross Pathology

All the Sunshine virus positive snakes from the veterinary diagnostic laboratory cases were in good to excellent body condition with moderately-sized to large coelomic fat bodies. Gross pathological findings were largely unremarkable and limited to mild or moderate pulmonary congestion and oedema in four snakes and fibrinonecrotic exudate adherent to the oral mucosa in one snake.

### 7.4 Histopathology

The most consistent histological lesions of Sunshine virus positive snakes were in the brain. All cases exhibited mild to severe spongiosis of primarily the white matter of the hindbrain (Figures 7.2 and 7.3). In a minority of cases, the spongiosis also involved white matter tracts of the midbrain or the parenchyma of the cerebellum. In snakes with severe histological lesions, spongiosis and rarefaction of the parenchyma of the hindbrain extended the complete dorso-ventral height of the tissue and thus involved the intermingled grey matter (Figure 7.3). In three of these severely affected snakes, neuronal chromatolysis or necrosis was evident in the hindbrain (Figure 7.3 inset). Mild to marked gliosis, composed of both astrocytosis and microgliosis, generally accompanied the spongiosis (Figures 7.2 and 7.3) and in four cases extended anteriorly to a lesser degree into the grey matter of the forebrain and olfactory bulb. Severely affected areas contained necrotic cell debris and low numbers of Gitter cells,
primarily located in the meninges and surrounding parenchymal blood vessels. Lymphoplasmacytic perivascular cuffing and meningeal infiltration were prominent in only one snake. Axonal swellings and Wallerian degeneration were uncommon in the hindbrain. Intracytoplasmic eosinophilic or pale basophilic inclusion bodies were rarely observed in astrocytes, ependymal cells and the epithelium of the choroid plexus and in most cases deemed equivocal. The tinctorial properties of the inclusions tended to vary in the brain and in other tissues with the relative strength of the eosin or haematoxylin staining in the particular slide. In the two snakes in which cervical spinal cord was examined histologically, the tissue was normal in one snake while the other snake, which also had prominent hindbrain lesions, exhibited moderate spongiosis of the cervical white matter with accompanying Wallerian degeneration.

Figure 7.2 Dorsal hindbrain, Berrimah Veterinary Laboratories, Collection A, jungle carpet python 1. Moderate patchy spongiosis involving primarily the white matter of the hindbrain (HB) and to a mild degree, the granular layer of the cerebellum (CB). Note mild degree of associated gliosis and lack of inflammatory cellular infiltrate in the meninges (M). CP= choroid plexus in the fourth ventricle. Haematoxylin and eosin stain. Bar = 200 µm.
Figure 7.3 Brainstem, Berrimah Veterinary Laboratories, Collection B, carpet python 2. Severe spongiosis involving the complete dorsoventral height of the hindbrain (HB) with relative sparing of the cerebellum (CB), midbrain (MB, including optic tectum (OT)), cranial extent of the spinal cord (SP) and caudal extent of the forebrain (FB). The rectangle outlines the area of the inset in which moderate gliosis and neuronal necrosis (arrowheads) are evident. Haematoxylin and eosin stain. Main bar = 500 µm. Inset bar = 50 µm.

Five snakes exhibited changes indicative of mild to moderate bronchointerstitial pneumonia, variably including pulmonary septal and/or faveolar oedema or mild heterophil infiltration, mild to moderate hyperplasia, erosion or necrosis of the luminal respiratory epithelium with associated patchy lymphoplasmacytic infiltration (Figure 7.4). One snake exhibited moderate diffuse type 2 pneumocyte hyperplasia. Rare equivocal intracytoplasmic inclusions were present in the respiratory epithelium, particularly in areas of eroded or necrotic epithelium. The most convincing possible viral inclusions were observed in the distal renal tubular system (primarily collecting ducts) in two snakes (Figure 7.5). In the five snakes in which spleen was examined, notable findings were mild to moderate lymphoid depletion in two snakes, marked lymphoid hyperplasia in one snake and a few small parenchymal heterophilic granulomas in another snake. All snakes exhibited a mild to moderate degree of macrovesicular vacuolation
of hepatocyte cytoplasm (hepatic lipidosis, which is a common finding in captive snakes and considered incidental). Other miscellaneous histological findings were mild renal tubular degeneration or interstitial fibrosis in two snakes, mild to moderate colonic heterophil infiltration in two snakes and necrotising stomatitis in one snake. All other organs examined were histologically unremarkable.

Figure 7.A Lung, Berrimah Veterinary Laboratories, Collection A, carpet python. Mild hyperplasia and jumbling of the luminal respiratory epithelium (LRE) with scattered necrotic cells and associated moderate lymphoplasmacytic infiltration. Generalised moderate vascular congestion and mild septal (S) oedema. The few red blood cells in the air spaces (faveolae) between septae are interpreted as post-mortem artefact rather than pre-mortem haemorrhage. Haematoxylin and eosin stain. Bar = 50 µm.
Published descriptions of the brain pathology associated with previously described paramyxoviruses in snakes are limited, with most publications focusing on the lung pathology since respiratory signs typically predominate in the infections (Jacobson et al., 1981; Jacobson et al., 1997; Kolesnikovas et al., 2006; Jacobson, 2007). However, in the few publications where the brain histology is reported, lesions are variably described as absent, ballooning degeneration and demyelination of the brainstem, degeneration of axon fibres, gliosis, lymphohistiocytic meningoencephalitis, neuronal degeneration and few eosinophilic intracytoplasmic inclusions in glial cells (Jacobson et al., 1980b; Jacobson et al., 1992; West et al., 2001). Thus the brain lesions seen in these cases associated with Sunshine virus infection include many of the histological features of other paramyxoviral infections in snakes. Aspects of the brain lesions in the cases described in this report that may emerge with further study as particular features associated with Sunshine virus versus other snake paramyxoviruses are the predominance of lesions involving the hindbrain and their severity. From a comparative

Figure 7.5 Renal collecting duct, Berrimah Veterinary Laboratories, Collection A, jungle carpet python 3. Basophilic intracytoplasmic inclusion bodies in epithelial cells (arrows). Haematoxylin and eosin stain. Bar = 10 µm.
pathology standpoint it is notable how similar the brain lesions associated with Sunshine virus infection are to those due to canine distemper virus in which the brainstem is frequently the most severely affected region and white matter lesions of spongiosis (intramyelenic oedema) may predominate early in the infection with eventual development of nonsuppurative inflammation, demyelination and the presence of Gitter cells (Summers et al., 1995; Caswell and Williams, 2007; Zachary, 2007).

The respiratory lesions in snake paramyxovirus infections and canine distemper virus are described as bronchointerstitial pneumonia with hyperplasia of type 2 pneumocytes and variable neutrophilic/heterophilic and/or lymphocytic infiltrates, commonly with superimposed secondary bacterial bronchopneumonia (Jacobson et al., 1992; Jacobson et al., 1997; Kolesnikovas et al., 2006; Caswell and Williams, 2007; MacLachlan and Dubovi, 2011). In the present cases of Sunshine virus, bronchointerstitial pneumonia was present in most cases although lesions were fairly mild, suggesting that Sunshine virus may be relatively less pneumotropic and more neurotropic compared to these other paramyxoviruses. Paramyxovirus infections may result in intracytoplasmic or intranuclear eosinophilic inclusion bodies in neurons, glial cells and a variety of epithelia, notably those of the respiratory and urinary systems (Caswell and Williams 2007, MacLachlan and Dubovy 2011, West et al 2001). In only a few cases of Sunshine virus infection were possible intracytoplasmic eosinophilic inclusions seen in the glial cells and respiratory epithelium, and those were considered equivocal since they occurred in areas of severe inflammation and could have represented fragments of necrotic cells or tissue, rather than viral inclusions. The most distinct intracytoplasmic inclusions seen in these Sunshine virus cases were in the otherwise normal renal distal tubule and collecting duct epithelia. Future work should include transmission electron microscopy of the inclusions seen in Sunshine virus, to determine if they are viral in nature.
Along with paramyxoviral infection, inclusion body disease (IBD) is another infectious disease of snakes that is capable of causing neurological signs (Schumacher et al., 1994; Jacobson et al., 2001b; Vancraeynest et al., 2006; Jacobson, 2007; Chang and Jacobson, 2010). Neurohistopathology associated with this syndrome includes diffuse spongiosis, demyelination, neuronal degeneration, gliosis and nonsuppurative or lymphoplasmacytic meningoencephalitis (Schumacher et al., 1994; Carlisle-Nowak et al., 1998; Jacobson et al., 2001b; Vancraeynest et al., 2006). The histopathology associated with Sunshine virus infection overlaps somewhat with that associated with IBD. However, in most cases of IBD, bright eosinophilic intracytoplasmic inclusions are readily appreciable within neurons and in abundance in epithelial cells in a variety of tissues (Schumacher et al., 1994; Carlisle-Nowak et al., 1998; Chang and Jacobson, 2010), a feature that was not present in the Sunshine virus cases detailed here.

Reoviruses have been either identified by electron microscopy or isolated from various snakes, some with clinical signs of neurological dysfunction (Ahne et al., 1987b; Vieler et al., 1994; Rose et al., 2005; Abbas et al., 2011). Unfortunately, the brain was not examined histologically in these reports, therefore any neuropathology that is associated with reovirus infection remains undescribed. Finally, an intranuclear inclusion disorder has been described in Morelia sp. that was associated with writhing and bloating (Boyer et al 2000). Electron microscopy of the brain revealed accumulations of particles that resembled retroviruses. However, the defining histological lesion in this syndrome was abundant prominent eosinophilic or amphiphilic intranuclear inclusion bodies in glial cells in the brain (described in (Boyer et al., 2000) and images reproduced in (Ritchie, 2006)), inconsistent with the rare, equivocal intracytoplasmic inclusions in glial cells in these Sunshine virus cases.
7.5 Chapter Summary

The development of a PCR that can detect Sunshine virus in a range of clinical samples has allowed infected animals to be rapidly identified. Consequently, clinical data that is associated with infection can be obtained. PCR testing was performed on freshly-frozen samples of brain, lung, kidney and liver, formalin-fixed paraffin-embedded (FFPE) tissues, serum, combined oral-cloacal swabs, cloacal-only swabs and oral-only swabs. In total, 141 samples from 48 snakes from six collections were tested for Sunshine virus by PCR. From these, 42 samples were positive: 25 freshly-frozen samples (samples from eight brains, seven kidneys, five lungs and five livers), six FFPE tissues and 11 swabs.

A number of conclusions could be drawn from the results of the PCR testing described in this chapter. They are summarised as follows:

1. The PCR described is capable of detecting Sunshine virus in freshly-frozen tissue samples, FFPE material and oral and cloacal swabs,
2. Sunshine virus was detected most often in samples of brain, followed by (in order) kidney, liver and lung,
3. PCR was able to identify snakes infected with Sunshine virus more often than virus isolation,
4. Virus isolation remains an important component of virus discovery as it is not reliant on previously determined sequence information,
5. It is likely that Sunshine virus will be detected more commonly in tissue samples than swabs,
6. Sunshine virus was not detected in serum samples,
7. There is a poor correlation between infection with Sunshine virus and the presence or type of clinical signs. Clinical signs are not always associated with Sunshine virus but when they are present they are most commonly non-specific (e.g. regurgitation, lethargy),
neurological (e.g. opisthotonus, head tremors) or neurorespiratory (e.g. viscous clear discharge from mouth, open mouth gaping).

8. Not all snakes infected with Sunshine virus face imminent death,

9. Serial testing of swabs is recommended as Sunshine virus may not be detected in the first swab sample, and

10. Sunshine virus has so far only been detected in pythons.

Dr Cathy Shilton, an anatomical veterinary pathologist from the Berrimah Veterinary Laboratories (BVL) in the Northern Territory described the gross pathology and histological findings associated with Sunshine virus infection. Gross pathology is usually unremarkable but pulmonary congestion has been seen occasionally. The most consistent histological lesions exhibited by snakes that are infected with Sunshine virus occur in the hindbrain and are characterised by white matter spongiosis and gliosis with extension to the surrounding grey matter and neuronal necrosis evident in severe cases. Most infected snakes also exhibit mild bronchointerstitial pneumonia.
Chapter 8 – Thesis Summary and General Discussion

In 1972, an outbreak of neurorespiratory disease in a Swiss collection of 446 snakes formed the basis for the first description of a paramyxovirus isolated from a snake (Folsch and Leloup, 1976). In the forty years that has passed since this outbreak, there have been approximately 50 published reports describing the paramyxoviruses found in other similarly-affected reptiles. Reptilian paramyxoviruses have been found in several European countries (Ahne et al., 1987a; Blahak, 1995; Manwell et al., 2000; Franke et al., 2001), USA (Jacobson et al., 1980b; Jacobson et al., 1981; Potgieter et al., 1987; Richter et al., 1996) and Brazil (Nogueira et al., 2002; Kolesnikovas et al., 2006). These paramyxoviruses have only ever clustered within the proposed genus Ferlavirus (Marschang, 2011) and prior to the work presented here, reptilian paramyxoviruses had not been reported in Australia. Not only does this thesis provide the first description of a paramyxovirus isolated from an Australian snake but this paramyxovirus, named Sunshine virus, is novel and only distantly related to the ferlaviruses.

8.1 Thesis Summary

In Australia, snakes displaying neurorespiratory signs have been presented to Australian veterinarians for at least the last few decades (Johnson, 2011). Histopathological examination of some of these snakes has shown interstitial pneumonia and demyelinating nonsuppurative encephalopathy, consistent with, but not pathognomonic for, a paramyxoviral infection. In some instances, the attending veterinarian has pursued agent-specific diagnostic tests available only in USA or Europe. Testing formalin-fixed paraffin-embedded tissues by in situ hybridisation and freshly-frozen serum by haemagglutination inhibition provided results that were suggestive of a ferlaviral infection. It has been shown that there was considerable discordance between the HI results provided by three American laboratories (Allender et al., 2008). These Australian snake samples were only submitted to one of these laboratories and furthermore, paired serum samples (where the second sample is collected after a period of convalescence) were not submitted. This means that the HI results could not be viewed as
irrefutable evidence of the presence of ferlavirus in Australia. In brief, the histological findings and diagnostic test results in Australian symptomatic snakes had provided a justification for an investigation of these snakes to find a scientifically-supported explanation for their clinical signs.

Australian veterinarians from six of Australia’s eight states and territories submitted 463 samples that had been collected from 192 snakes from 36 collections from May 2007 to October 2009. 217 samples were analysed using a haemagglutination assay, five were analysed by a neuraminidase assay, 86 by a haemagglutination inhibition assay, 50 by polymerase chain reaction (PCR) and 161 by virus isolation. In 2007, during an outbreak of neurorespiratory disease in a Queensland zoological collection, two cloacal swabs from two asymptomatic snakes were collected that were found to contain haemagglutinating and neuraminidase activity. Further testing by electron microscopy (EM) and PCR did not identify a virus. In 2008, during a separate outbreak of neurorespiratory disease, this time in a private Queensland collection of pythons, a virus was isolated from two pythons: a black-headed python (BHP1) with low grade respiratory signs and stomatitis, and a jungle python with subtle neurological signs. The cytopathic effect (CPE) in viper heart (VH2) cells primarily consisted of syncytial cell formation. Viruses with similar CPE in VH2 cells were also isolated from freshly-frozen samples that were submitted by Dr Cathy Shilton, an anatomical veterinary pathologist from the Northern Territory. One of these viruses from the Queensland outbreak, BHP1-Lung, was selected for further investigation.

Despite widespread and repeatable CPE, the identity of BHP1-Lung (the viral family it belonged to) escaped discovery for over a year despite utilising a suite of traditional virological methods: positive and negative staining transmission electron microscopy of infected VH2 cells and supernatant respectively; haemagglutination and haemadsorption assays; neuraminidase activity; and the effect on viral titre of a DNA synthesis inhibitor (5-bromo-3-deoxyuridine),
acid, chloroform and ether. The results of this testing were either negative or equivocal and the low viral titre (TCID$_{50} = 10^{2.75}$ mL$^{-1}$) was believed to have contributed to at least some of these results. The identity of BHP1-Lung could also not be determined by PCR using primers designed to detect a broad range of paramyxoviruses, reoviruses, adenoviruses and herpesviruses.

Eventually, BHP1-Lung was identified as a novel paramyxovirus using high-throughput sequencing. This method provides sequence-independent amplification of a sample without requiring prior knowledge about the identity of the virus being investigated. 10,544,936 reads (each 38 nucleotides in length) were sequenced and assembled into 5,818 unique contigs. A batch search of GenBank using BLASTN (Basic Local Alignment Search Tool for Nucleotides) provided a result that contained the word “virus” for 326 of these contigs. The open reading frames (ORFs) were determined for each of these contigs and only one contained ORFs with sequence similarity to a paramyxovirus. Further analysis of this contig was able to identify six ORFs common to most paramyxoviruses: 3’ nucleocapsid (N) – putative phosphoprotein (P) – matrix (M) – fusion (F) – putative attachment protein – polymerase (L). This analysis was unable to classify the putative attachment protein as haemagglutinin-neuraminidase (HN), haemagglutinin (H) or glycoprotein (G). Phylogenetic analysis of BHP1-Lung performed by Dr James Wellehan placed this virus within Paramyxoviridae but outside of existing subfamilies. BHP1-Lung was named Sunshine virus after the geographical origin of the first isolate, the Sunshine Coast of Queensland. Several paramyxoviruses have been named this way: Menangle virus, Newcastle disease virus, Hendra virus, Nipah virus, Beilong virus, Sendai virus, Mossman virus and Salem virus.

Sequence information of Sunshine virus that had been determined from high-throughput sequencing allowed PCR primers to be designed that could rapidly identify this virus in a variety of clinical samples. The ability to identify animals infected with Sunshine virus would
allow the clinical signs, gross pathology and histological findings that are associated with this infection to be reported. The samples provided by Dr Cathy Shilton along with those from the submission that produced BHP1, and a third set of samples from an outbreak of neurological disease from Queensland, were tested for Sunshine virus by PCR. In total, 141 samples (71 freshly-frozen tissues, 47 swabs, 13 serum samples and ten FFPE sections of various tissues) from 48 snakes from six collections were tested and from these, 42 samples were positive. This newly-designed PCR had been able to detect Sunshine virus in freshly-frozen brain, liver, kidney and lung; FFPE sections; and cloacal and oral swabs. The virus could not be detected in 13 serum samples.

Dr Cathy Shilton described the pathological findings associated with Sunshine virus infection. Gross pathology was usually unremarkable and the most consistent histopathology was seen in the hindbrain as white matter spongiosis and gliosis with extension to the surrounding grey matter and neuronal necrosis evident in severe cases. Mild bronchointerstitial pneumonia was seen in most infected snakes.

The work provided in this thesis describes a novel paramyxovirus named Sunshine virus that was isolated from Australian snakes and is often associated with neurorespiratory clinical signs and pathology. Although a considerable volume of useful information is provided in this thesis, it has only provided an introduction to a new virus that is associated with disease.

8.2 The Aims of the Project

The broad aims of this thesis were listed in the first chapter (section 1.1 – Purpose of Project) but can now be reviewed to see if each aim was met.
1. To isolate and identify a virus from Australian snakes that could explain the clinical signs, pathological findings and overseas diagnostic test results that have been suggestive but not conclusive of a paramyxovirus infection.

To achieve this aim, a virus would need to be isolated (and identified) from Australian snakes that could explain the clinical signs, pathological findings and the overseas diagnostic test results that had been recorded in these animals that were all consistent with a paramyxoviral infection. Discussed earlier in Chapter 3 – Paramyxoviruses in Australian Snakes, the clinical signs recorded in many of these snakes can be localised to the neurorespiratory systems, the gross pathology is often unremarkable, the histopathology often describes a demyelinating encephalopathy and anti-ferlaviral antibodies and ferlaviral nucleic acid has been detected by haemagglutination inhibition and in situ hybridisation, respectively, in overseas laboratories.

Samples from 192 snakes were screened for the presence of a range of viruses and nine viruses were isolated from five snakes (Table 5.3). One of these isolates was analysed further and was identified as a novel paramyxovirus named Sunshine virus. The clinical signs and pathological findings that were associated with Sunshine virus infection are described in Chapter 7 – Diagnosing Sunshine virus. The clinical signs associated with Sunshine virus can often be localised to the neurorespiratory systems (section 7.2 – Clinical Signs). Additionally, the gross and histopathological findings seen with Sunshine virus infection are also consistent with the changes described in Chapter 3. However, the results of overseas diagnostic testing are difficult to explain if the animals that were tested were infected with Sunshine virus and not ferlavirus.

Significant titres (> 8) of anti-ferlaviral antibody could not be detected in the serum samples from a private collection of pythons, infected with Sunshine virus, during an outbreak of neurorespiratory disease (Figure 5.18). This may have been because the snakes in this
collection had not developed a detectable immune response to ferlavirus because they were not infected with this virus and secondly, because there is no cross-reactivity between Sunshine virus and the ferlavirus used in this antibody assay. So the presence of Sunshine virus in some Australian snakes does not explain the presence of anti-ferlaviral antibodies in other snakes. However, the possibility that the significant anti-ferlaviral antibody titres of the snakes that were tested overseas were in fact false results, should not be excluded. The results of this study are unable to provide further clarification.

Some Australian snake samples were sent overseas where in situ hybridisation (ISH) was positive for ferlavirus. It is possible that these results were false positives where the probe annealed to a target that was not ferlavirus but assuming these results were true then the presence of Sunshine virus in some Australian snakes is not able to explain this result in other Australian snakes.

In this project, 86 serum samples were tested for the presence of anti-ferlaviral antibodies. These samples were from snakes with neurorespiratory disease or from snakes that were in direct or indirect contact with affected snakes. Significant titres were not found in any sample (0/86). Testing for ferlavirus by polymerase chain reaction (PCR) in 50 samples from similarly affected snakes also failed to detect the presence of ferlavirus (0/50). These results are in stark contrast to the results of overseas testing where significant titres ( > 8) of anti-ferlaviral antibodies were found in 52 out of the 65 samples from similarly affected snakes (52/65, Table 3.1). These positive anti-ferlaviral antibody titres are supported by ISH results. Ferlaviral nucleic acid was detected in samples from some snakes that had previously tested positive for the presence of anti-ferlaviral antibodies. If it is assumed that all these sets of results are true, then ferlavirus is present in Australia and this project was unable to detect any evidence of it in 50 samples suitable for PCR testing and 86 serum samples. If the ferlaviral results of this project are false and the results of overseas testing are true than ferlavirus is present in
Australia and the screening for ferlavirus that was performed in this project is inappropriate for the detection of ferlavirus. In the final hypothetical situation, where the results of this project are true and the results of the overseas testing are false, there is no unequivocal evidence for the presence of ferlavirus in Australia but in at least some of the Australian snakes with clinical signs and pathological findings consistent with a paramyxoviral infection, Sunshine virus is one possibility to explain these findings. Future studies testing the positive and negative predictive values of the testing regimes used in both this project and those that are used overseas would be needed to provide further insight into the validity of these results.

In conclusion, this aim was partially met as the isolation and identification of Sunshine virus is explanatory for the neurorespiratory clinical signs and pathological findings in at least some Australian snakes but is not explanatory for the results of overseas diagnostic testing. This aim was partially achieved.

2. To investigate if the association between disease and the presence of this isolated virus, is causative.

The results of this project have demonstrated that Sunshine virus is explanatory for the clinical signs and pathological findings (that were consistent with a paramyxoviral infection) seen in at least some Australian snakes. However, this project does not provide evidence that Sunshine virus was the cause of these findings. To meet this aim, a transmission study is needed. This was not performed due to time and financial restraints. This aim was not achieved and should form a priority of future studies and is discussed in greater detail on page 264.

3. To produce a diagnostic test that could rapidly screen Australian snakes for the presence of this virus.
Following the isolation of Sunshine virus from Australian snakes and identifying it as a unique paramyxovirus that is only distantly related to the proposed genus *Ferlavirus*, it became clear that testing for the presence of ferlavirus would not be adequate to detect the presence of Sunshine virus. A PCR that could rapidly identify Sunshine virus was designed, produced and validated on positive control material and a range of clinical samples (section 6.5 – Development and Validation of a Sunshine virus Polymerase Chain Reaction Primer Set). This aim was achieved.

4. To apply this diagnostic test to Australian snake samples to provide clinically useful information about the biology of this viral infection e.g. shedding patterns, tissue predilections.

141 samples were screened using a PCR primer set that was produced for its capability to rapidly screen samples for the presence of Sunshine virus. 42 of these samples were found to be infected with Sunshine virus and by identifying these positive samples, clinically useful information about the biology of Sunshine virus infection could be described. Ten specific conclusions were drawn from this testing (page 255). However, this list of conclusions is far from complete and there is still a considerable amount of information to be revealed. For example, the onset and duration of Sunshine virus shedding has not been fully elucidated. This aim was partially achieved.

There is still a large amount of important research that should follow this project to extend the findings discovered so far. Some directions that this research may take are discussed.

### 8.3 Future Directions of Research

**Transmission Study, Immunohistochemistry and In situ Hybridisation**

Investigations should be pursued that might help determine if Sunshine virus has a causative association with disease. Although the author is unaware of any non-pathogenic
Paramyxoviruses and a paramyxovirus is explanatory for the clinical signs and histopathological findings reported here, a causative link between Sunshine virus infection and disease has not yet been proven. A transmission study would provide an opportunity to demonstrate Koch’s postulates. This has already been reported for two other pathogenic snake viruses: ferlavirus (Jacobson et al., 1997) and a reptilian orthoreovirus (Lamirande et al., 1999).

A transmission study could also provide important information about disease pathogenesis. The onset, nature and duration of clinical signs could be observed. Also, by serially sampling and testing oral and cloacal swabs for the presence of Sunshine virus, the onset and duration of viral shedding from each site could be determined. This would have direct relevance to diagnosing this disease. For example, if it was discovered that Sunshine virus had a very short shedding period, it could be unwise to serially collect and test swabs for the presence of virus over an extended period of time. As another example, if it was discovered that the onset of clinical signs and/or viral shedding did not occur for several months, this information could form the basis for extended quarantine period recommendations in situations where agent-specific testing is not performed.

Serially sampling and testing serum could reveal the onset and duration of viremia. Finally, during such a study, the animal’s environment (e.g. water bowls, hideboxes etc) could be swabbed and tested for the presence of Sunshine virus. This could provide insight into the environmental persistence of this virus. All these results would provide valuable information that could improve the ability to diagnose and manage cases of Sunshine virus infection.

Immunohistochemistry (IHC) and in situ hybridisation (ISH) can demonstrate if an antigen or nucleic acid, respectively, is temporally and spatially associated with histological lesions. Put more simply, these methods could demonstrate that Sunshine virus (or parts of the virus) was present in histological lesions (a spatial association) at the time of death (a temporal
association). These methods can help delineate a causal association from one that is non-causal. Using Sunshine virus that had been purified by density gradient ultracentrifugation would allow both monoclonal and polyclonal antibodies to be produced that could be used in IHC. These assays (IHC and/or ISH) could potentially identify the cell types and intracellular locations where virus is commonly found. These assays could also be used diagnostically.

**Reviewing the Sunshine Virus Polymerase Chain Reaction**

The non-degenerate primer sets for the detection of Sunshine virus that are described in this thesis should be reviewed. This is particularly important considering the minimal sequence variation that has been identified in the sequenced amplicons to date. Although the minimal sequence variation may be explained by the highly conserved part of the paramyxoviral genome (the polymerase gene) that these primers target (Kurath et al., 2004), the ability of these primer sets to detect significant differences between different strains of Sunshine virus has not been demonstrated and so the application of these primers for diagnostic use may be limited. Degenerate primers may broaden the detection capabilities for Sunshine virus, possibly revealing closely-related viruses.

Robust controls should also be incorporated into this diagnostic PCR. In addition to positive and negative control samples, the integrity of the RNA in the test samples should be able to be assessed by this assay. Using the transcripts of housekeeping genes (e.g. 18S rRNA, beta-actin) may provide some insight into the quality of the viral RNA. Additionally, spiking positive controls (that can be differentiated from true positives) into each test sample would control for each nucleic acid extraction. Following a positive PCR result, consideration should also be given to a restriction enzyme digestion to provide a method of positive conformation that is faster than sequencing.

The PCR designed for the detection of Sunshine virus that is described in this thesis is being offered to Australian veterinarians as a diagnostic tool. It is hoped that the ability to detect
Sunshine virus will allow the health of Australian snakes to be better managed. Only by screening samples with these primers will pitfalls in the PCR be able to be detected.

In the not-too-distant future, the Sunshine virus PCR will be applied to free-ranging snakes to investigate the possibility that this virus is present in non-captive animals.

**Humoral Assays**

The ability to screen snakes for an immune response to Sunshine virus infection would complement PCR-testing. A PCR test can only detect a virus if there is viral nucleic acid in the sample, so if the duration of Sunshine virus shedding was brief, testing swabs for the presence of this virus may not detect all infected animals. In contrast, the presence of viral nucleic acid is not needed to detect circulating anti-virus antibodies. However, humoral assays are reliant on these circulating anti-virus antibodies being present in detectable quantities at the time of sampling and this requires a period of convalescence to have elapsed. For Sunshine virus, the length of this period of convalescence (the time to seroconvert) is not known but might be determined during a transmission study.

For other reptilian viruses, humoral assays (e.g. haemagglutination inhibition and virus neutralisation) are available at a small number of diagnostic laboratories (Heard et al., 2004). Although a single antibody titre will not always reliably distinguish between prior and current exposure to viral antigen (especially if the sample could contain both IgY from IgM), it may still help to decide the fate of animals, especially those that are PCR-negative where it is unclear if the PCR result is merely a reflection of an animal that is no longer shedding virus.

Subsequent to the work presented in this thesis, Sunshine virus has been sent to Dr Rachel Marschang in Germany for future studies. Initially, this virus will be used for antibody assays (virus neutralisation) in an investigation to see if there is immunological evidence for the presence of Sunshine virus in Europe.
**Treatment and Vaccination**

The isolation of Sunshine virus into cell culture allows *in vitro* testing of antivirals and also the propagation of virus that could be used in vaccines. Anti-paramyxoviral agents such as ribavirin (Virazole®) and BCX 2798 (reviewed on page 26) would be reasonable candidates to assess the impact that they may have on the viral titre of Sunshine virus. Screening anti-viral agents in an *in vitro* environment may decrease the number of animals needed for *in vivo* studies. The development of a reptilian vaccine that protects against a virus has not yet been achieved but lessons learnt from previous attempts (Jacobson et al., 1991; Lloyd and Flanagan, 1991; Marschang et al., 2001b) could help to develop a vaccine that protects against Sunshine virus infection. A DNA vaccine may provide a novel opportunity to develop a vaccine that cannot revert to virulence but still provides protective immunity.

**8.4 Conclusion**

This thesis has described the isolation of a novel paramyxovirus named Sunshine virus from Australian snakes. A set of PCR primers was subsequently designed to facilitate the detection of this new virus in clinical samples. This PCR has enabled clinical data that is associated with Sunshine virus infection to be described. Future research directions have been clearly identified that will hopefully continue to contribute to the collective knowledge of reptilian viruses for many years to come.
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Appendix

Molecular Detection of Two Adenoviruses Associated with Disease in Australian Lizards

T Hyndman1 and CM Shilton2

We give the first published description of the pathology and molecular findings associated with adenovirus infection in lizards in Australia. A central notched dragon (Ctenophorus nuchalis) exhibited severe necrotising hepatitis with abundant intercellular inclusion bodies within hepatocytes and rarely within intestinal epithelial cells. Polymerase chain reaction (PCR) using pooled tissues yielded an amplicon that shared strong nucleotide identity with an agaravid adenovirus (EU914203). PCR on the liver of a bearded dragon (Pogona minor minor) with ichthyosis, coccidiosis, nocardiosis and hepatic lipodosis yielded an amplicon with strong nucleotide identity to a herpethocordi adenovirus (EU914207).

Keywords adenoviruses; Australian lizards; polymerase chain reaction; reptiles

Abbreviations PCR, polymerase chain reaction

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Adenoviruses are non-enveloped, double-stranded DNA viruses known to infect every class of vertebrate.1,2 Adenoviral or adenoviral-like infections have been found in lizards,3,4 snakes,5,6 chelonians and crocodiles.7 The liver and gastrointestinal tract are two commonly affected anatomical sites. Reptilian adenoviruses have been detected in the USA,1 Europe1,2,8 and New Zealand, but to date there have not been any published reports from Australia. A nested polymerase chain reaction (PCR) primer set based upon the consensus sequence data from a conserved region of the DNA-dependent DNA-polymerase gene from diverse members of the family Adenoviridae has allowed the rapid molecular detection of adenoviruses from a range of samples.2 We describe the discovery of adenoviruses from two genera of Australian lizards.

Materials and methods

All tissue samples submitted for histopathological examination were processed using standard techniques. DNA was extracted from fresh frozen tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Doncaster, Vic, Australia) according to the manufacturer’s guidelines. For DNA extraction from formalin-fixed paraffin-embedded material, a fresh microtome was used for each block to cut 1-4 unstained 6-µm sections that were then placed into a single 2-ml microcentrifuge tube. The QiAamp DNA FFPE Tissue Kit (Qiagen) was then used according to the manufacturer’s instructions to extract the DNA from the tissue sections. Using the MagMAX Viral DNA Isolation Kit (Ambion, Austin, TX, USA), canine adenovirus type 2 DNA was extracted from a canine vaccine (Protech C3, Fort Dodge, Auckland, New Zealand). The lysing/binding buffer was injected into the vaccine vial to dissolve the freeze-dried powder. The resultant solution was then removed and processing of the sample continued according to the manufacturer’s guidelines. Canine adenovirus was used as a positive control for the adenovirus PCR.

Extracted DNA was then used in an adenovirus PCR that has previously been used for the successful identification of reptilian adenoviruses. Briefly, 1 µl of extracted DNA, used as a template, was added to 18 µl of Platinum® PCR SuperMix (Invitrogen, Mulgrave, Vic, Australia). PolF (5’TNNCMGNGNMMNTGTYACCA) and PolR (5’GTDCGACANHCMCKFBARRATNHGTT) were used at final concentrations of 3 µM each. The final reaction volume was 20 µl. Cycling conditions in a thermal cycler (2720 Thermal Cycler, Applied Biosystems, Scoresby, Vic, Australia) consisted of an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min and elongation at 72°C for 45 s, followed by a final elongation phase at 72°C for 5 min. The PCR product (1 µl) was used as template in a second round of PCR using the primer set PolF (5’GTNTRYGATHTTCGCGGATGATGG) and PolR (5’ CCNGGCGCCDGTTGGTGCCA), Conditions for this round of PCR were identical to the first.

PCR products were separated using 2% agarose gel (w/v), in Tris-acetate-EDTA buffer with 0.005% (v/v) SYBR safe; Invitrogen) electrophoresis at 80 V for 80 min and visualised using a transilluminator (DR886M, Dark Reader non-UV Transilluminator; Clare Medical Research, Dolores, CO, USA). Bands of the correct size were excised from the gel using a sterile scalpel blade, purified using the Purufix Quick Gel Extraction Kit (Invitrogen) and then directly sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Primer sequences were excluded from the amplicons, which were then compared to the GenBank database (www.ncbi.nlm.nih.gov/genbank/index.html). Samples that produced an amplicon of the appropriate size after the nested PCR were then re-amplified in a semi-nested PCR using the primers PolF and PolR in the first round and PolF and PolR in the second round. This process enabled more than 100 bases of additional sequence information to be retrieved from the samples.

Results

The signalement and history for each case are summarised in Table 1, as are the gross and histopathological findings. Adenovirus PCR using
Table 1. Summary of findings in two Australian lizard species with adenovirus infection

<table>
<thead>
<tr>
<th>Species, location (year)</th>
<th>Clinical history</th>
<th>Gross pathology</th>
<th>Histopathology</th>
<th>Adenovirus PCR assay</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central netted dragon (Ctenophorus nuchalis), Darwin, NT (2006)</td>
<td>Recently acquired from another captive collection, prior history and age unknown, found dead in enclosure with no premonitory clinical signs</td>
<td>Diffusely mottled red/maroon liver (Figure 1), haemorrhage in intestinal lumen</td>
<td>Severe diffuse necrotising hepatitis with moderate numbers of large amphiphilic intranuclear inclusion bodies in hepatocytes (Figure 2), occasional amphiphilic intranuclear inclusions in large intestinal mucosal epithelial cells (Figure 3)</td>
<td>Positive on formalin-fixed paraffin-embedded material</td>
<td>No intestinal pathology to suggest cause of intraluminal haemorrhage, possibly a coagulopathy resulting from diffuse hepatic necrosis</td>
</tr>
<tr>
<td>Western bearded dragon (Pogona minor minor), Perth, WA (2009)</td>
<td>Juvenile with lithritht, hepcacomegaly, coecidiosis, nematodiasis, euthanased. Three in contact captive with similar clinical signs.</td>
<td>No cellular inclusions or evidence of hepatic necrosis or inflammation</td>
<td>Hepatic lipidosis</td>
<td>Positive on fresh-frozen liver</td>
<td>Only liver submitted for analysis</td>
</tr>
</tbody>
</table>

NT, Northern Territory; PCR, polymerase chain reaction; WA, Western Australia; +ve, positive.

To the best of our knowledge, this is the first published description of the molecular detection of reptilian adenoviruses in Australia. Knowledge of the status of reptilian viral diseases in Australia is limited to only a handful of references and only a few have identified these pathogens using more specific methods such as electron microscopy and/or molecular methods. It is our hope that our findings will alert Australian practitioners who deal with reptilian patients to the possibility of adenovirus infection.

Several studies have identified adenovirus infections in bearded dragons. The signalment of many lizards infected with adenovirus is biased towards juveniles and, commonly, ill-thrift and concurrent infections, such as coccidiosis and/or nematode parasites have been found. Hepatic necrosis was the main finding in one of the cases presented here, consistent with many other reports. Therefore, a juvenile dragon with a history of ill-thrift, with or without other pathogens (such as coccidiosis or nematodes), or with evidence of hepatic necrosis or hepatitis should raise the suspicion of adenovirus infection in the clinician’s mind. In the P. minor minor in which adenovirus was detected in the liver, there was no histological evidence of hepatic necrosis or hepatitis, so the role of the adenovirus in this animal’s condition remains undefined.

The primer set used in this investigation has been recommended for the discovery of ‘new’ adenoviruses because of its exceptional sensitivity across all current genera of Adenoviridae. At the time of writing, the PCR primer set that was used in this study is being
used in at least two laboratories (in Florida, USA and Germany) for the diagnosis of adenoviruses from reptilian clinical samples. Exporting samples from Australia to international laboratories for diagnosis is problematic because various governmental permits need to be obtained (e.g., from the Convention on the International Trade of Endangered Species, the Australian Quarantine and Inspection Service and State or Territory wildlife management agencies). Furthermore, it is becoming increasingly difficult for many overseas laboratories to satisfy the legal requirements for the importation of international samples. Therefore, it is valuable that this adenovirus PCR is available in Australia. Not only will this provide a service that is more accessible to Australian veterinarians but turnaround times for results will also be improved.

In the USA, most inland bearded dragons (Pogona vitticeps) that are PCR-tested for adenovirus are positive (P. Wellchan, University of Florida, pers. commun.). By enabling rapid and specific diagnosis of adenoviruses to be made in Australia, the ability to screen both wild and captive Australian reptiles for infectious diseases will be improved. More informed decisions can then be made concerning the healthcare of these animals.

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