Discovery of Herpesviruses and other Potential Pathogenic Viruses in Australian Wildlife

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Declaration of Ethics

The research presented and reported in this thesis was conducted with the approval of the James Cook University Research Ethics Committee and in accordance with the National Statement on Ethical Conduct in Human Research, 2007; Australian Code for the Care and Use of Animals for Scientific Purposes, 2007; and the Queensland Animal Care and Protection Act, 2001. The proposed research methodology received clearance from James Cook University Experimentation Ethics Committee (A2587). This research was conducted under permits granted by the Department of Environment and Science (WA0012830).

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Statement of Contribution of Others

This research was supervised by A/Prof Paul F. Horwood, Prof David Whitmore and Prof Ellen Ariel. They contributed to the research conceptualization and provided extensive feedback on all of the written works in this thesis. Paul and Ellen contributed to research design, execution, sample collection, validation, and result interpretation. Paul, Ellen, and David contributed to funding acquisition and provided editorial support to this thesis. This thesis was proofread by Dr Diana Mendez.

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List of Abbreviations

aa	amino acid
ACDP	Australian Centre for Disease Preparedness
AdV	adenovirus
AITHM	Australian Institute of Tropical Health and Medicine
AwAdV	agile wallaby atadenovirus
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
ChelHV	chelid herpesvirus
ChHV5	chelonid alphaherpesvirus 5
CODEHOP	Consensus Degenerate Hybrid Oligonucleotide Primer
DMSO	dimethyl sulfoxide
DPOL	DNA polymerase
FP	Fibropapillomatosis
gB	glycoprotein B
HV	herpesvirus
ICTV	International Committee on Taxonomy of Viruses
JCU	James Cook University
LNA	locked nucleic acid
LOD	limit of detection
ML	maximum likelihood
n	number
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
nt	nucleotide
NTC	no template control
ORF	open reading frame
PCR	polymerase chain reaction
SsTAdV	saw-shelled turtle adenovirus
STC-PCR	singleplex touchdown consensus PCR
TMAC	tetramethyl ammonium chloride
VIDR	Infectious Diseases Reference Laboratory

Abstract

The past several decades have witnessed a steady increase in the emergence of novel viruses in wildlife species globally. These novel emerging viruses have been recognised as potential threats to wildlife and human populations. In Australia, novel viruses have continued to emerge and threaten the conservation of wildlife species, as exemplified by the fatal outbreak caused by the Bellinger River virus that almost led to the extinction of the Bellinger River snapping turtle (*Myuchelys georgesi*). Also, a herpesvirus (HV) was suspected to be the cause of a cutaneous lesion outbreak in a freshwater population in 2016 at Alligator Creek, Queensland, Australia. Several pathogenic viruses including HVs, picornaviruses, and orbiviruses have been reported to cause fatal epizootic diseases in Australian marsupials. Marsupials including agile wallabies are highly abundant wildlife species in Australia and are known to be competent reservoirs of zoonotic viruses. Their abundance as well as increased human interactions pose zoonotic concerns. The aim of this study was to identify and characterise novel herpesviruses and other potential pathogenic viruses in freshwater turtles (*Myuchelys latisternum* and *Emydura macquarii krefftii*) and agile wallabies (*Notamacropus agilis*) using broad-based viral discovery tools such as consensus PCR and next generation sequencing.

Rapid and sensitive assays are vital for the early detection of novel pathogenic viruses, which would consequently aid managerial decisions on conservation strategies. In this thesis a sensitive singleplex touchdown PCR (STC-PCR) assay was developed to investigate disease outbreaks and monitor HV emergence (viral discovery) in wildlife species. The STC-PCR was successfully tested against a wide variety of HV DNAs in two independent laboratories and showed good sensitivity when compared to a commonly used assay.

As a result, the STC-PCR was deployed in combination with other consensus PCR assays for the robust investigation of viral occurrence in the suspected diseased population of freshwater turtles. A total of six novel viruses; alphaherpesviruses (chelid herpesvirus 1-3) and testadenoviruses (saw-shelled turtle adenovirus 1-3), were discovered for the first time in any species of Australian freshwater turtles. The novel herpesviruses detected in this study were closely related to the tumour associated chelonid alphaherpesvirus 5. However, statistical analysis using odds ratio showed that there was no association between the novel herpesviruses and cutaneous lesions.

Furthermore, metagenomic and consensus PCR were used in a complementary manner to investigate the occurrence of potential pathogenic viruses harboured by agile wallabies. A total of 14 novel viruses belonging to the family *Adenoviridae*, *Parvoviridae*, *Polyomaviridae*, *Circoviridae*, and *Picobirnaviridae* were identified in the fecal samples of free-ranging agile wallabies. Phylogenetic analyses indicate that these novel viruses would have co-evolved with their hosts (agile wallabies). A known macropod herpesvirus 3 was also detected. This study highlighted the usefulness of integrating broad-based assays for monitoring the emergence of potential pathogenic viruses in wildlife populations.

Phylogenetic analysis is not sufficient in predicting the pathogenic potential of novel viruses, and the lack of specific cell culture or occurrence of unculturable viruses could hamper further characterisation of some important novel viruses. These shortcomings could lead to the under-reporting of potential pathogenic or zoonotic viruses, and ultimately limit our understanding of the clinical, epidemiological and conservation significance of novel viruses. Therefore, an effective workflow for the discovery and reporting of novel viruses in wildlife species have been proposed in this thesis.

Overall, the discoveries and approaches used in this study have significant implications for the design and implementation of wildlife conservation and disease monitoring plans. Also, wildlife conservation strategies would benefit from the important recommendations made in this thesis.

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Chapter One: General Introduction

Australian wildlife is made up of a wide variety of animals including mammals, birds, reptiles, and amphibians, many of which are unique to the continent (Cresswell & Murphy, 2017). The Australian wildlife is characterized by a huge abundance of reptiles, marsupials, mammals and birds occupying a vast majority of ecological niches (Cresswell & Murphy, 2017), and they regularly exist with humans in semi-urban situations or along the edges of larger cities. In north Queensland, it is considered a daily occurrence to see wallabies scattering from the lawns on campus in the mornings, to hear the "plop" of a turtle as it drags into the water from its basking spot, to have a python in the attic to keep down the rodent population, and to be woken by kookaburras and noisy parrots. The wildlife in Australia constitutes an important aspect of national heritage as well as playing a vital role in maintaining healthy ecosystems, serving as source of food, and contributing to environmental aesthetics (Everett, 1979; Stephen et al., 2018). However, a significant proportion of wildlife species in Australia are considered threatened and this is due to several factors including climate change, natural disasters, urbanisation, introduced non-native species, and diseases (Allek et al., 2018; Stobo-Wilson et al., 2021). In the past, these threats have contributed to the extinction of some wildlife species including the paradise parrot (*Psephotus pulcherrimus*), southern pig-footed bandicoot (*Chaeropus ecaudatus*), and the broad-faced potoroo (*Potorous platyops*) (Woinarski et al., 2019).

In the past few decades, there has been a steady increase of disease outbreaks in wildlife populations worldwide caused by emerging novel viruses (Cunningham et al., 2017; Daszak et al., 2000; Hyatt et al., 1997; Roelke-Parker et al., 1996; Sarker, 2022). Viral diseases are recognised as an increasing threat to wildlife and a major cause of important clinical diseases of humans due to factors such as habitat destruction, pollution, and international trade (Chiu,

2013; Cunningham et al., 2017; Sarker, 2022). Most emerging viruses of pandemic potential (e.g., Ebola, Marburg virus, monkey pox virus, influenza viruses, retroviruses, and coronaviruses) originate from wildlife and spill over to human hosts (Letko et al., 2020; Reperant & Osterhaus, 2017; Sarker, 2022). Many vulnerable or endangered wildlife populations in Australia are still susceptible to highly virulent viral pathogens that can potentially cause extinction of extant species, as exemplified by the Bellinger River snapping turtle (*Myuchelys georgesi*) population in 2015 (McCallum, 2012; Raidal et al., 2015; Timm et al., 2009). Therefore, it has become pertinent to monitor the health of wildlife populations for the timely detection of novel and emerging viral pathogens, which in turn would facilitate the implementation of useful intervention or conservation strategies.

During a routine health monitoring in 2016, cutaneous lesions were observed in a large number of freshwater turtle species (*Emydura macquarii krefftii* and *Myuchelys latisternum*) in Alligator creek, North Queensland, Australia (Wirth et al., 2020). Preliminary investigation implicated herpesvirus (HV) as a potential causative agent, however, further investigation was needed to understand the role of HV in the disease aetiology and pathogenesis. Herpesviruses consist of a large group of enveloped DNA viruses belonging to the family *Herpesviridae* (Győző L Kaján et al., 2020; McGeoch et al., 2006). The family *Herpesviridae* is divided into three subfamilies, namely *Alpha*-, *Beta*- and *Gamma-herpesvirinae* (Cohen, 2010; Davison, 2010). Alpha-HVs have a wide host range and rapid replication whereas beta- and gamma-HVs have relatively narrow host range and longer growth cycles (Cohen, 2010). Although HVs have the capacity to cause life-long infections and remain latent in adaptive hosts, they can cause severe diseases when reactivated by factors such as stress, co-infections, immunosuppression, or host naivety (MacLachlan & Dubovi, 2011; Sehrawat et al., 2018). In Australia, HVs have been detected in different wildlife species including birds, reptiles, and marsupials (Amery-Gale et al., 2018; Jones et al., 2020; Langhorne et al., 2021; Phalen et al.,

2011; Shilton et al., 2016; Stalder et al., 2015), however, some of these viruses are not well characterised, and there is a dearth of epidemiological data, which limits our understanding of the impacts of these viruses and possible control measures. These challenges could be attributed to the lack of efficient disease monitoring tools that can detect a broad range of novel and emerging viruses in wildlife populations.

Wildlife disease investigations face different challenges including inaccessibility of samples or sampling sites of free-ranging species, and adverse environmental conditions (Baily, 2016). This can be further complicated by the ethical requirements of invasive sampling as well as the technical difficulties associated with expedient sample transport and storage (Baily, 2016; Zemanova, 2019). One way to overcome some of these challenges is through the development and implementation of non-invasive sampling methods whenever possible. For instance, it is almost impossible to trap free-ranging agile wallabies (*Notamacropus agilis*) without subjecting them to a great deal of stress. However, non-lethal, easily accessible fresh fecal samples belonging to agile wallabies are available on the lawn around campus in the early mornings and these samples can be screened for the presence of potential pathogenic and zoonotic viruses with appropriate methods as part of disease monitoring plans.

Current available diagnostic techniques are generally narrow in scope and have limited application in the investigation of novel and emerging viral pathogens (Chiu, 2013; Kiselev et al., 2020). Traditional assays such as culture, serology, or specific polymerase chain reaction (PCR) are selectively sensitive and have limited usage where there is no *a priori* knowledge of the potential pathogenic agents (Chiu, 2013; Kiselev et al., 2020; Yang & Rothman, 2004). In contrast, multiple pathogen detection tools such as consensus PCR (with degenerate primers) and next generation sequencing (NGS) are capable of identifying a wide range of known and novel viruses in clinical samples, thus making them suitable for disease

monitoring and pathogen discovery studies in wildlife populations (Bird & Mazet, 2018; Chiu, 2013; Datta et al., 2015). However, currently available consensus (universal) PCR assays for HV detection are still marred by low or selective sensitivity and specificity, and discovery is limited to only viruses within the family (Chiu, 2013; Yang & Rothman, 2004). Similarly, detection of non-isolated viruses from clinical samples by NGS is dependent on the amount present as low abundant viruses are mostly missed (Datta et al., 2015; Houldcroft et al., 2017; Nurlan Sandybayev et al., 2022). Therefore, it is imperative to improve upon currently available diagnostic tools as well as consolidate the broad-based approaches for efficient disease surveillance and reporting, which would consequently improve the decision platform for managerial practice and thereby strengthen conservation efforts in Australia.

This study aims to discover novel viruses in freshwater turtles and agile wallabies residing in North Queensland using broad-based assays such as consensus PCR and NGS with a view to provide data and new approaches for use in conservation policies or studies relating to the health of wildlife populations in Australia. The main objectives of this study are to:

1. systematically review literature and assess information relevant to the study of herpesviruses and other potential pathogenic viruses in reptiles and marsupials (Chapter 2).
2. develop subfamily-based consensus PCR assays for the discovery of herpesviruses (Chapter 3).
3. identify and characterise novel herpesviruses and other pathogenic viruses in freshwater turtle species using consensus PCR assays (Chapter 4).
4. identify and characterise novel viruses of free-ranging agile wallabies by the complementary use of metagenomics (NGS) and consensus PCR approaches (Chapter 5).

5. propose an effective workflow for viral discovery and disease monitoring in wildlife (Chapter 6).

While preparing the works presented in this thesis, I aimed to published during the process where possible. This has so far resulted in three publications and two manuscripts under review (Table 1.1).

Table 1. 1. Publications resulting from this thesis.

Publication	Status	Relevant to chapter
Okoh, G.R., Horwood, P.F., Whitmore, D. and Ariel, E. (2021). Herpesviruses in Reptiles. *Frontiers in Veterinary Science*, 8:642894. Doi: https://doi.org/10.3389/fvets.2021.642894	Published	2
Okoh, G.R., Ariel, E., Whitmore, D. and Horwood, P.F. (2023). Viruses Infecting Australian Marsupials: A Scoping Review	In progress	2
Okoh, G.R., Lockhart, M., Grimsey, J., Whitmore, D., Ariel E., Butler, J. and Horwood P.F. (2023). Development of Subfamily-Based Consensus PCR Assays for the Detection of Human and Animal Herpesviruses. *European Journal of Clinical Microbiology and Infectious Diseases*. Doi: https://doi.org/10.1007/s10096-023-04605-w	Published	3
Okoh, G.R., Ariel E., Wirth, W., Whitmore D. and Horwood P.F. (2023). Molecular	Published	4

Detection of Novel Herpesviruses and Adenoviruses in Two Species of Australian Freshwater Turtles. *European Journal of Wildlife Research*. Doi: https://doi.org/10.1007/s10344-023-01714-x		
Okoh, G.R., Ariel, E., Whitmore, D. and Horwood, P.F. (2023). Metagenomic and Molecular Detection of Novel Fecal Viruses in Free-ranging agile Wallabies. *EcoHealth*.	In review	5
Okoh, G.R., Ariel, E., Whitmore, D. and Horwood, P.F. (2023). Draft Genome Sequence of a Novel Adenovirus Recovered from the Metagenome of Agile Wallabies. *Microbiology Resource Announcements*, e00112-23. Doi: https://doi.org/10.1128/mra.00112-23	Published	5

Chapter Two: Literature Review

2.1. Background and Aims

There is a paucity of epidemiological data that limits our understanding of the impacts of herpesviruses and other pathogenic viruses on the health of Australian wildlife. Chapter two consists of two reviews that focus on identifying knowledge gaps relevant to the study of herpesviruses and other potential pathogenic viruses in reptiles and marsupials.

In 2016, herpesvirus was suspected to be the cause of a disease outbreak (cutaneous lesions) in freshwater turtles at Alligator Creek, Queensland (Wirth et al., 2020). To further investigate this disease outbreak, I conducted a review to equip myself with the current knowledge on the clinical and epidemiological features of herpesvirus infections in reptiles. The first part of this chapter reviewed available literature (1972-2020) on herpesviruses infecting reptiles and aimed to provide updated information on the taxonomy, epidemiology, diagnosis, prevention, and control of reptilian herpesviruses. This review was published in 2021 and included relevant information on reptilian herpesviruses at the time of publication. This part of the chapter has been updated to include a section that summarises the current knowledge on reptilian herpesviruses since the review was published (2021-2023). The second part of this chapter is an up-to-date review that provides evidence on the epidemiological status of viruses infecting marsupials in Australia; the knowledge gained from this review was subsequently applied to the investigation of novel viruses in agile wallabies.

Overall, the aim of this chapter was to systematically review literature to assess information on the status of viruses in reptiles and marsupials. To address this aim, this chapter describes:

1. the taxonomy of known and novel reptilian herpesviruses.

2. the clinical presentations of herpesviruses in reptiles.
3. the epidemiological implications of herpesviruses in reptiles.
4. the diagnostic approaches used for the detection of reptilian herpesviruses.
5. the occurrence, clinical and zoonotic significance of viruses in Australian marsupials.

2.2. Publications arising from this chapter

- **Okoh, G.R.,** Horwood, P.F., Whitmore, D. and Ariel, E. (2021). Herpesviruses in Reptiles. *Frontiers in Veterinary Science*, 8:642894. Doi: 10.3389/fvets.2021.642894
- **Okoh, G.R.**, Ariel, E., Whitmore, D. and Horwood, P.F. (in progress). Viruses Infecting Australian Marsupials: A Scoping Review.

2.3. Part I: Herpesviruses in Reptiles

2.3.1. Introduction

Reptiles are a group of vertebrates (class Reptilia) that are adapted to a broad range of terrestrial and aquatic environments (Cogger, 2014; Rasmussen et al., 2011). The group comprises over 11,000 extant species placed in four orders, namely: Testudines (turtles, tortoises, and terrapins); Squamata (lizards, snakes, and worm lizards); Crocodilia (crocodiles, alligators, gharials, and caimans); and Rhynchocephalia (tuatara) (Mans et al., 2021). Reptiles constitute an integral part of the natural ecosystem and play the roles of both pollinators and predators, as well as environmental health indicators (Gibbons et al., 2000). In addition to their ecological services, reptiles have become desirable for food, medicinal products, pet trade, leather goods and research applications (González et al., 2016; Robinson et al., 2015; Zug & Dowling, 2020). However, their existence and well-being have constantly been threatened by several factors, such as hunting, environmental pollution, loss of habitat, destructive non-native species, climate change, and infectious diseases (Gibbons et al., 2000; Groom et al., 2006; Todd et al., 2010; Van Der Ploeg et al., 2011). Disease surveillance and research in wild populations of reptiles are associated with numerous challenges including difficulties in accessing samples or field data, misleading epidemiological data and missing population data, as well as political and cultural restrictions (Ryser-Degiorgis, 2013). These challenges well explain the use of captive wildlife as models in many studies to acquire epidemiological information, since diseases are comparable in both wild and captive animals (Haines & Kleese, 1977; Hausmann et al., 2015; Marenzoni et al., 2018; Munson & Cook, 1993). Nonetheless, more robust and ideal epidemiological data are obtained when free ranging animals are surveyed. Recently, researchers have taken a renewed interest in reptilian viruses, partly due to the role played by reptiles as reservoir hosts for zoonotic viruses, as

well as improvements in viral diagnostic methods that, in turn, have increased understanding of viruses in reptiles (Ariel, 2011; Dahlin et al., 2016; Habarugira et al., 2020; Machain-Williams et al., 2013; Marschang, 2011).

Herpesviruses (HVs) are members of the family *Herpesviridae*, a large taxon of DNA viruses that have been described in most vertebrate animals, including reptiles (Ariel, 2011; Mettenleiter et al., 2008). Herpesviruses are enveloped viruses with an icosahedral nucleocapsid and a linear double-stranded genome of varying length from ~124 to 259 kbp (Payne, 2017). Generally, HVs replicate within host cell nuclei and can remain latent in their natural hosts (Jacobson, 2007; Marschang, 2011). So far, reptilian HVs that have been identified and characterised all belong to the subfamily *Alphaherpesvirinae* (Aplasca et al., 2019; Goe et al., 2016; Marschang, 2011; Sharma et al., 2016).

The occurrence of HV infections among reptiles has been widely documented and associated with stomatitis, tumors, encephalitis, conjunctivitis, hepatitis, and mortalities (Adamovicz et al., 2018; Jungwirth et al., 2014). Unfortunately, current treatment options of reptilian HVs are limited and the search for potent vaccines remains a herculean task; therefore, the adoption of preventative strategies is still the most efficient way of controlling these diseases. This review aims to assist in biosecurity planning as well as creating a knowledge platform for decision makers and researchers by providing an overview of the taxonomy, pathogenesis, pathology, and epidemiology of reptilian HVs.

2.3.2. Methods

Databases such as Medline (Ovid), PubMed, and Scopus were searched using specific keywords and phrases including Herpesviridae infections, herpesvirus infection, fibropapillomatosis, grey-patch disease, loggerhead genital-respiratory herpesvirus, herpesvirus disease, reptiles, turtles, tortoise, snakes, lizards, alligators, and crocodiles (see

Appendix 1: Supplementary Table 2.1). To ensure that relevant publications were not missed, each subheading was searched independently on PubMed. Also, an additional literature search was conducted by assessing references of articles selected from previous databases. A summary of the search results is shown in Figure 2.1. Furthermore, I read the abstracts and full texts of the selected articles, extracted and analysed information on the diagnostic methods used and the reptilian HVs investigated from 1972 to September 8, 2020 (Figures 2.2 and 2.3; Appendix 1: Supplementary Table 2.2). Non-English, non-original research, guidelines, and review articles were excluded from the analysis.

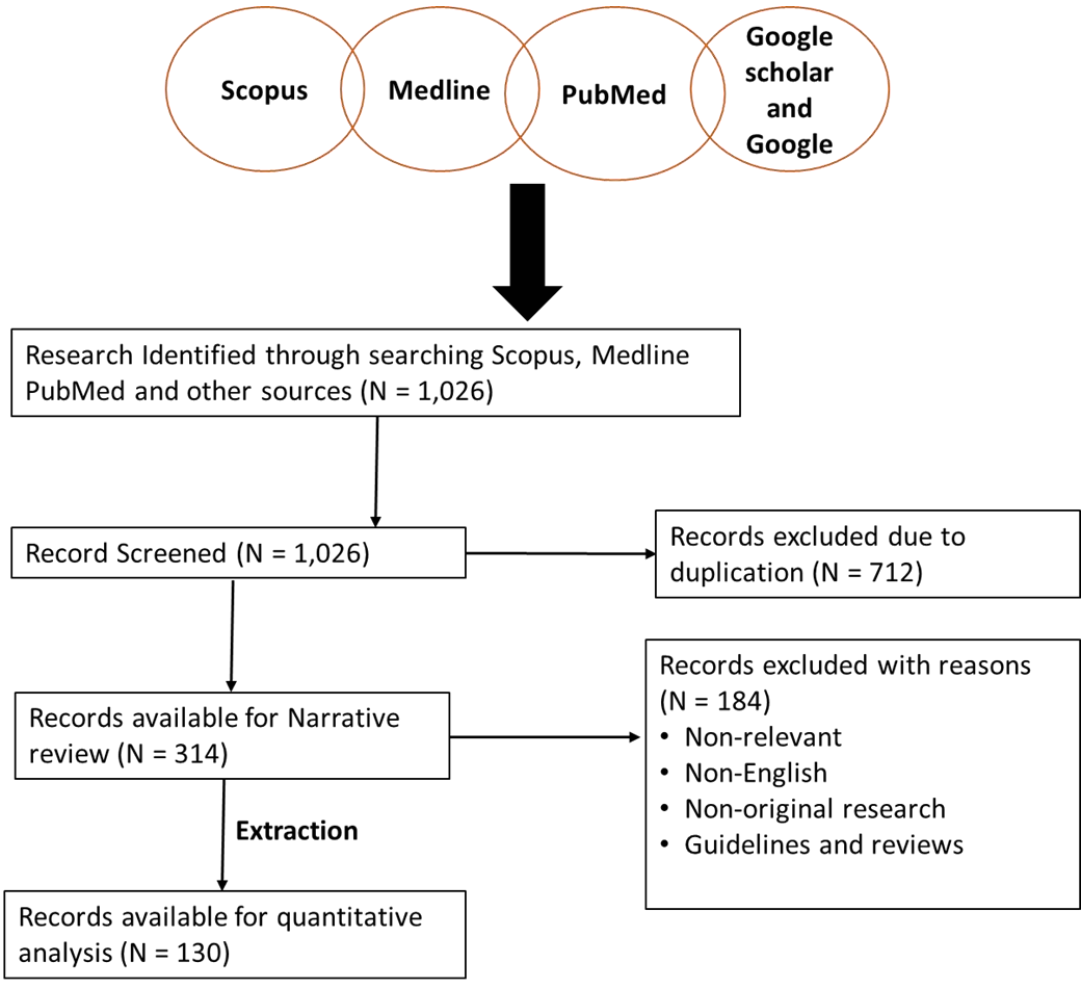


Figure 2. 1. Summary of literature search conducted. A total of 1,026 articles were initially screened and 712 articles were later excluded due to duplication. A total of 314 articles were

then reviewed for this study. One hundred and thirty studies were then extracted for quantitative analysis.

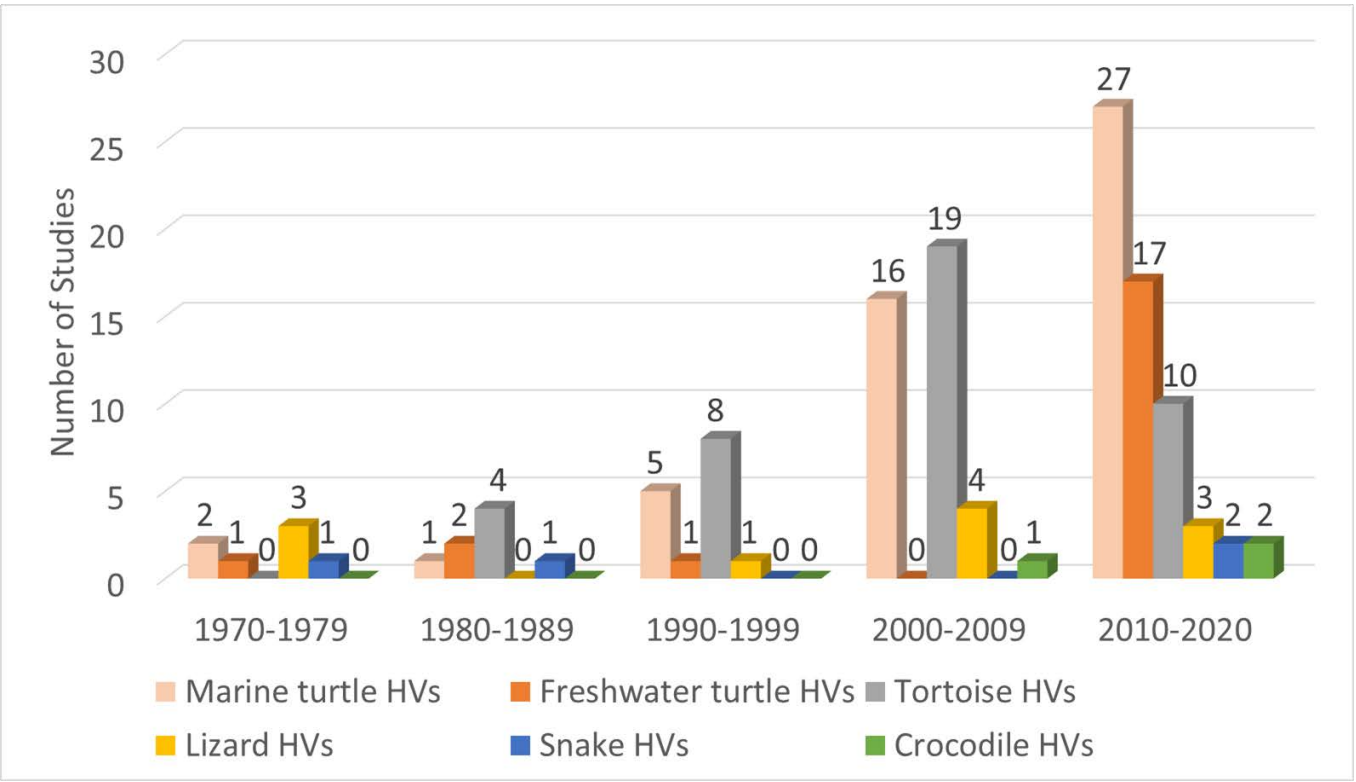


Figure 2. 2. Studies on HVs in reptiles from 1972 to September 8, 2020. Overall, more than a quarter (n = 51; 39%) of the 130 extracted studies were conducted in marine turtles since the 1970s. Of the 60 publications between 2010 and 2020, 45% (n = 27) were ChHV-5 related studies.

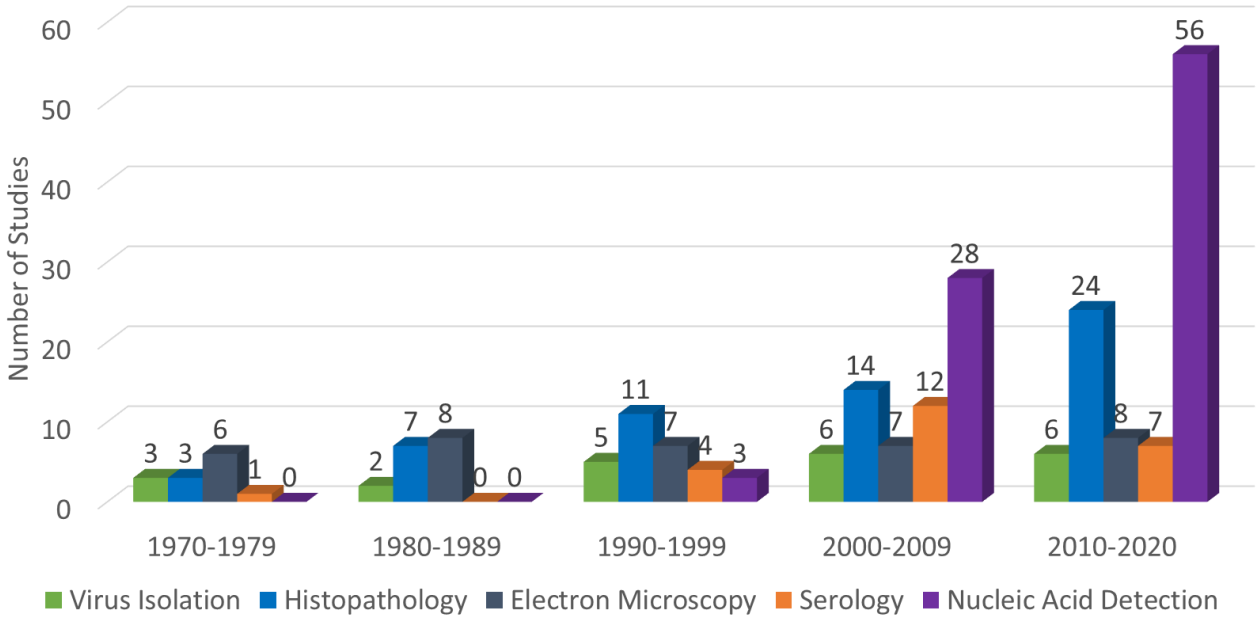


Figure 2. 3. Diagnostic methods used in different studies from inception 1970 to September 8, 2020. Of the 130 studies extracted for this quantitative analysis, 22 (17%), 59 (45%), 36 (28%), 24 (18%), and 87 (67%) used virus isolation, histopathology, electron microscopy, serology, and nucleic acid detection assays, respectively, for various investigations of reptilian HVs.

2.3.3. Bibliometrics

I conducted bibliometric analyses of published articles on the topic of reptilian HVs using Vosviewer software (van Eck & Waltman, 2010) and the Web of Science Core Collection database. A total of 245 publications were downloaded from Web of Science Core Collection database using the following search terms: herpesvirus, turtle, lizard, snake, tortoise, and crocodile. The strategy involved a combined use of the keywords, tags, and Boolean operators to create query sets as follows: ALL= (herpesvirus) AND ALL= (turtle* OR lizard* OR snake* OR tortoise* OR crocodile*) with no limitations. USA had the highest number of research outputs with 149 (60.8%) articles. This was followed by Germany (28; 11.4%) and Australia (26; 10.6%) (Figure 2.4; Table 2.1). Using Vosviewer, I visualised the major

keywords commonly used in the field of reptilian HVs and the link strengths between collaborating countries (Figure 2.4; Appendix 1: Supplementary Figure 2.1). Of note, a low number of records on reptilian HVs were observed for some countries (Indonesia, Mexico, and India) that have rich reptile diversity (Table 2.1) (Butler, 2019; Fischetti, 2018). Some of these countries also had little or no collaborations with the high research output countries (Figure 2.4), thus suggesting an under-reporting of reptilian HVs in these countries. Conversely, Germany has less reptile diversity with a higher number of records (Table 2.1). This observation could be attributed to the presence of established diagnostic resources or increased monitoring and reporting systems for reptilian diseases in the country.

2.3.4. Taxonomy of Reptilian Herpesviruses

Reptilian HVs belong to the family Herpesviridae, a member of the order *Herpesvirales* (International Committee on Taxonomy of Viruses, 2021). According to the 2021 International Committee on Taxonomy of Viruses (ICTV) classification, the subfamily *Alphaherpesvirinae* comprises five genera namely, *IItovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus*, and *Varicellovirus*. Only the genus *Scutavirus* contains species that cause HV diseases in reptiles and includes *Chelonid alphaherpesvirus 5* (ChHV-5) and *Testudinid alphaherpesvirus 3* (TeHV-3). The species *Chelonid alphaherpesvirus 6* (ChHV-6) belongs to the subfamily *Alphaherpesvirinae* with unknown generic placement. The Iguanid herpesvirus 2 (IgHV-2) that causes cytopathic infection in iguanids is of unknown generic and subfamilial placement (Gatherer et al., 2018; International Committee on Taxonomy of Viruses, 2021; Wellehan et al., 2003; Wilkinson et al., 2005).

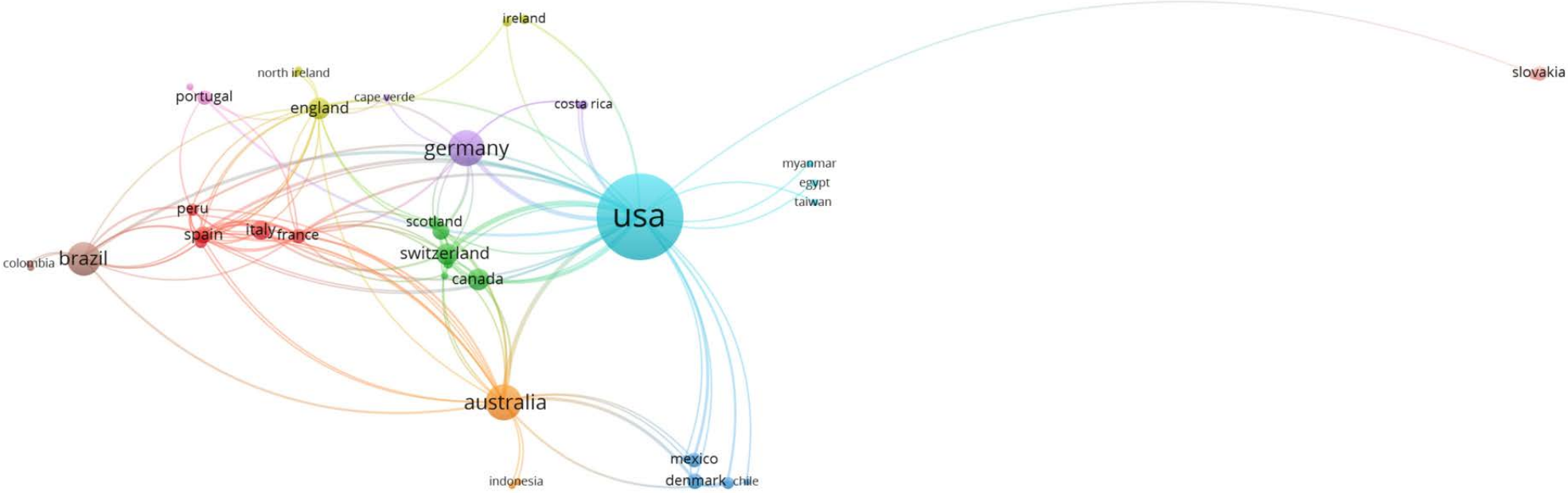


Figure 2. 4. Links between collaborating authors from different countries with research output from inception to 2020. The circular-coloured nodes represent countries, and the node size indicates number of publications from the country. The lines between nodes indicate authorship collaborations between countries and the widths of these lines indicate the link strength.

Table 2. 1. Bibliographic data on reptilian herpesviruses based on the number of articles from different countries.

Countries/Regions	**Records**	**% of 245**	**Number of reptile species (Butler, 2019; Uetz et al., 2022)**	***Rank**
USA	149	60.8	1147	2
Germany	28	11.4	29	31
Australia	26	10.6	1159	1
Brazil	23	9.4	878	5
Switzerland	11	4.5	27	32
Italy	10	4.1	65	24
Canada	9	3.7	57	25
United Kingdom	15	6.1	6	43
Belgium	6	2.4	11	41
Spain	6	2.4	78	23
Denmark	5	2.0	12	40
France	4	1.6	181	15
Mexico	4	1.6	1021	3
Portugal	4	1.6	44	27
Slovakia	4	1.6	16	37
Ecuador	3	1.2	541	10
Japan	3	1.2	111	22
China	3	1.2	605	8
Peru	3	1.2	586	9
Austria	2	0.8	18	36
Costa Rica	2	0.8	467	12
Czech Republic	2	0.8	15	38
India	2	0.8	889	4
Ireland	2	0.8	21	35
Netherlands	2	0.8	23	34

Barbados	1	0.4	13	39
Cape Verde	1	0.4	51	26
Chile	1	0.4	179	16
Colombia	1	0.4	654	7
Croatia	1	0.4	42	28
Egypt	1	0.4	133	20
French Guiana	1	0.4	177	17
Indonesia	1	0.4	798	6
Israel	1	0.4	148	19
Myanmar	1	0.4	374	13
Nicaragua	1	0.4	218	14
Norway	1	0.4	10	42
Romania	1	0.4	31	30
Seychelles	1	0.4	37	29
South Africa	1	0.4	529	11
South Korea	1	0.4	26	33
Taiwan	1	0.4	123	21
Turkey	1	0.4	150	18
Turks Caicos	1	0.4	12	40

*Ranking was conducted based on the number of reptile species by countries identified from my bibliometric search and not based on the global ranking by Butler (Butler, 2019).

Since the end of the twentieth century, advances in molecular and phylogenetic analyses have made it possible for novel reptilian HVs to be identified with proposed taxonomic placements (McGeoch & Gatherer, 2005). Novel HVs have been detected in freshwater turtles, including emydoidea herpesvirus 1 (EBHV-1), pelomedusid herpesvirus 1, glyptemys herpesvirus 1 and 2 (GlyHV-1 and−2), emydid herpesvirus 1 and 2 (EmyHV-1 and−2), and terrapene herpesvirus 1 and 2 (TerHV-1 and−2) (Jungwirth et al., 2014; Lindemann et al., 2018;

Marschang et al., 2015; Ossiboff, Raphael, et al., 2015; Sim et al., 2015; Yonkers et al., 2015). Loggerhead genital-respiratory herpesvirus (LGRV) and loggerhead orocutaneous herpesvirus (LOCV) were detected in loggerhead turtles (Caretta caretta) and the genus Chelonivirus was proposed for these viruses and other related chelonian HVs (Stacy et al., 2008). Also, tortoise HV species (TeHV-1-4) have been identified and placed in the proposed genus Chelonivirus (Bicknese et al., 2010; Johnson et al., 2005; Marschang, Frost, et al., 2001; Marschang et al., 2006), although TeHV-3 has been formally assigned to the genus *Scutavirus* (International Committee on Taxonomy of Viruses, 2021). Other unassigned reptilian HVs in the family *Herpesviridae* include the iguanid herpesvirus 1 (IgHV-1), gerrhosaurid herpesvirus 1-3, varanid herpesvirus (VHV-1,−2,−3) and helodermatid herpesvirus 1 (HeHV-1) in lizard species (Clark & Karzon, 1972; Goe et al., 2016; Hughes-Hanks et al., 2010; Wellehan et al., 2005; Wellehan et al., 2004; Zeigel & Clark, 1972), opheodrys herpesvirus 1 in snakes (Lovstad et al., 2019), crocodyline herpesvirus 1-3 (CrHV1-3) in crocodiles (Hyndman et al., 2015), and, chelonid herpesvirus 1-4 (ChHV1-4) in green turtles (*Chelonia mydas*-ChHV-1) (Rebell et al., 1975), freshwater turtles (*Clemmys marmorata*-ChHV-2; *Chrysemis picta*-ChHV-3) (Cox et al., 1980; Frye et al., 1977), and Argentine tortoise (*Geochelone chilensis*-ChHV-4) (E. Jacobson et al., 1985). Notably, some of these unassigned HVs were identified decades ago based on their morphological and biological characteristics using techniques (virus isolation, electron microscopy and histopathology) that were available at that time, thus making it challenging to place them taxonomically. I conducted a phylogenetic analysis to illustrate the relationship between the unassigned reptilian HVs and currently assigned HVs using amino acid sequences of HV-DNA-dependent DNA polymerase (37 complete and 17 partial sequences) from the NCBI website (https://www.ncbi.nlm.nih.gov/). As previously described (Davison, 2014; Ossiboff, Raphael, et al., 2015), the analysis showed that the unassigned reptilian HVs form a

monophyletic group with members of the subfamily *Alphaherpesvirinae*. Freshwater HVs showed a close phylogenetic relationship with the tortoise HVs while the lizard HVs indicated high variations (Figure 2.5). Overall, given the variations shown by the unassigned reptilian HVs, it remains a matter of scientific deliberation whether these viruses should be assigned into one genus such as TeHV-3 and ChHV-5 or into different genera, although I envisage the latter would be the case.

2.3.5. Virion and Genome Organization

All members of the family *Herpesviridae* share a common virion architecture, comprising a monopartite, linear, double stranded DNA core enclosed within an icosahedral capsid with a T =16 symmetry (Bowman et al., 2003; Caspar & Klug, 1962; Richard, 1996). The capsid is tightly wrapped by a proteinaceous tegument, which, in turn, is surrounded by an envelope containing polyamines, lipids, and essential antigenic glycoproteins (Richard, 1996; Roizmann et al., 1992). Unlike reptilian HVs, the atomic structures of human alphaherpesviruses have mostly been described. For instance, a cryo-electron microscopy (Cryo-EM) resolved the atomic structure of human simplexviruses (HSV-1 and−2), which comprise capsid organisation (hexons, pentons and triplexes), capsid proteins (VP5, VP19C, VP23, and VP26) and tegument proteins (pUL17, pUL25, and pUL36) (Brown & Newcomb, 2011; Dai & Zhou, 2018; Yuan et al., 2018). Although the atomic structures of reptilian HVs have not specifically been resolved, the resolved structures of other alphaherpesviruses provide insights, since their genomes have many similarities (McGeoch & Gatherer, 2005; Mocarski Jr, 2007). Consequently, the insights could guide future research in the atomic structure resolutions of reptilian HVs, which in turn could serve as a baseline for reptilian HV vaccinology.

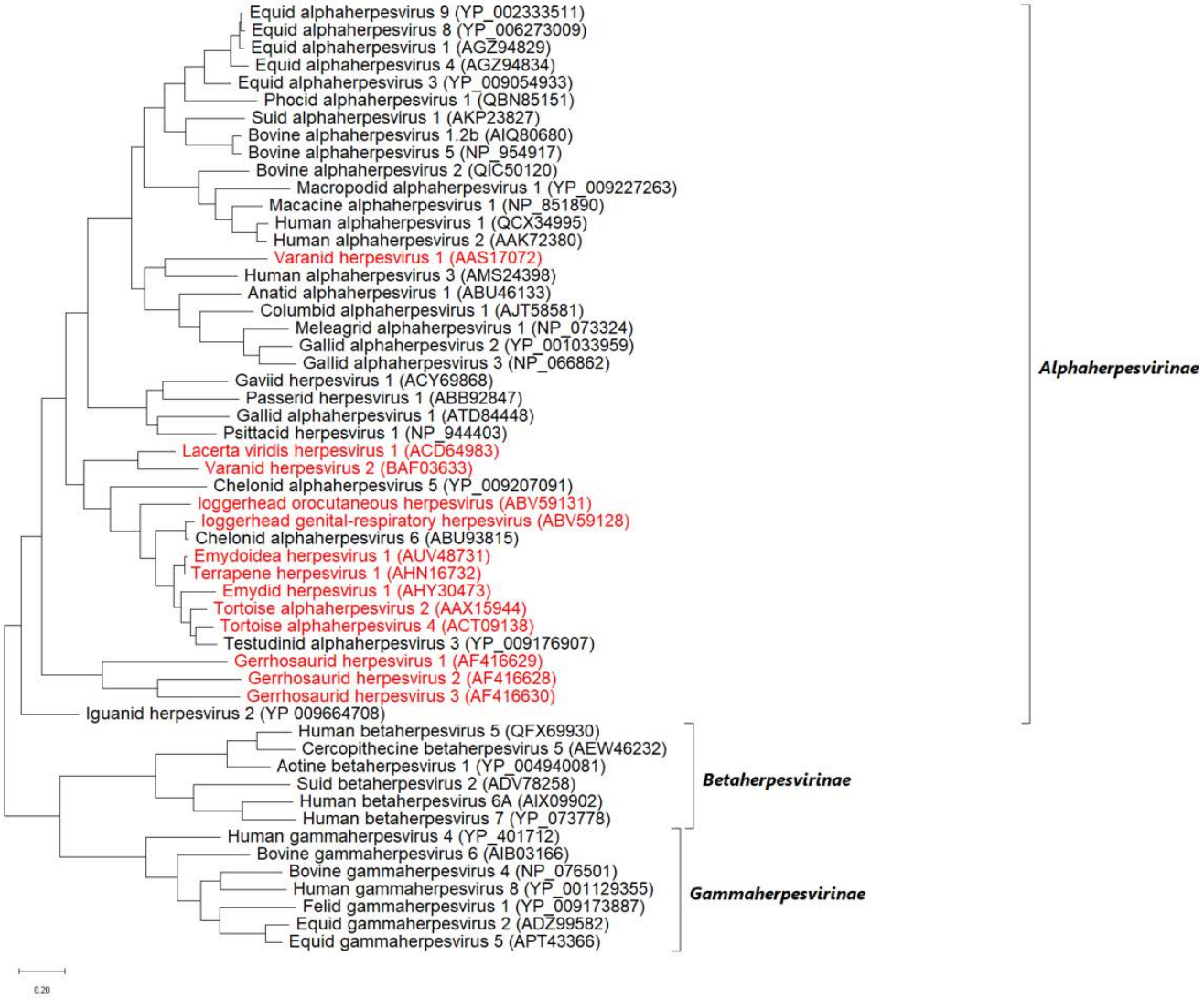


Figure 2. 5. Midpoint rooted maximum likelihood phylogenetic tree of predicted amino acid sequences of HV-DNA-dependent DNA polymerase. The unassigned reptilian HVs are shown in red and cluster within the subfamily *Alphaherpesvirinae*. This analysis involved 54 amino acid sequences (37 complete and 17 partial sequences) downloaded from NCBI website (https://www.ncbi.nlm.nih.gov/) and aligned by ClustalW. There was a total of 1,443 positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The tree with the highest log likelihood (−60139.09) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated

using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X.

Although the complete nucleotide sequences for most reptilian HVs are yet to be obtained, genomic features can be inferred from other fully sequenced alphaherpesviruses owing to sequence homology (McGeoch & Gatherer, 2005; Mocarski Jr, 2007). All alphaherpesvirus genome structures contain unique long (UL) and short (US) sequences and each are flanked by both terminal (TRL, TRS) and internal (IRL, IRS,) inverted repeat regions, giving the general configuration TRL-UL-IRL-IRS-US-TRS (Hay & Ruyechan, 2007). The complete genome of two TeHV-3 strains (1976 and 4295) was recently sequenced. The 1976 strain was shown to have a novel inverted repeat (TRT, IRT) and unique (UT) regions (Gandar et al., 2015). The genome is approximately 160 kbp, encodes at least 107 open reading frames (ORFs) and consists of UL (107,928 bp) and US (20,375 bp) regions. The UL is bound to its right by the US adjoined to inverted repeats (IRS and TRS; 8,536 bp) and to its left by a third unique region (UT; 12,595 bp), which is also bordered by inverted repeats (TRT and IRT; 1,194 bp) to give the overall layout TRT-UT-IRT-UL-IRS-US-TRS (Gandar et al., 2015). However, this differs from the type D configuration earlier attributed to this species (Origgi et al., 2015). In another study, the complete nucleotide sequence of a Bacterial Artificial Chromosome (BAC) containing the entire genome of ChHV-5 (cloned in pTARBAC2.1) was obtained and it showed a different configuration (UL-IRS-US-TRS) from that of TeHV-3, even though they both belong to the genus Scutavirus (Ackermann et al., 2012). Moreover, the genome characterisation of strain 4295 identified regions containing genes that could be involved in viral pathogenesis or virulence (Gandar et al., 2015). This is an important finding as these regions could serve as therapeutic or diagnostic targets in future research. Similarly, evidence of recombination among strains of ChHV-5 documented by Morrison et al. could

lead to increased virulence and transmission of ChHV-5 variants (Morrison et al., 2018), and these events may remain undetected in sea turtle populations. Therefore, it is pertinent to strengthen current diagnostic approaches to allow for more comprehensive geographical surveys and characterisation of HVs. Also, as new and affordable diagnostic techniques are being developed and improved upon, I expect more novel structures of reptilian HVs to be reported.

2.3.6. Transmission and Pathogenesis

Several modes of reptilian HV transmission have been postulated including vertical, horizontal, and mechanical transmissions (Jones et al., 2016; Müller et al., 1990; O'Rourke & Lertpiriyapong, 2015). Marenzoni et al. reported the first evidence of vertical transmission of TeHV-3 in a captive breeding facility (Marenzoni et al., 2018). In this study, one hatchling born in isolation from the egg laid by an infected tortoise (*Testudo hermanni hermanni*) presented with conjunctivitis and tested positive by specific polymerase chain reaction (PCR) targeting the partial sequence of the UL39 gene of TeHV-3 (Marenzoni et al., 2018). In other studies, Jones and colleagues provided molecular evidence for the horizontal transmission of ChHV-5 in green turtles by demonstrating the molecular link between viral variants and foraging grounds (Ariel et al., 2017; Jones et al., 2020). Furthermore, experimental studies have revealed the possible transmission of reptilian HVs by direct contact with infectious secretions (Chaves et al., 2017; Monezi et al., 2016; Origgi et al., 2004) or indirectly via vectors and water (Curry et al., 2000; Greenblatt et al., 2004; Herbst et al., 1998; Lu, Yu, et al., 2000). By linking viral shedding patterns or frequency to disease occurrence, we could trace the most probable transmission mode of reptilian HVs. For instance, in captive enclosures, HVs could be easily transmitted via contact with secretions or contaminated materials, even at low shedding rates, and the removal of infected animals and infectious materials could stop the spread of the virus. In the wild, different transmission agents such as

vectors, fomites and superspreaders could interplay to compensate for the low contact rate and infrequent shedding of some HVs (Work et al., 2015). Because of the managerial implications, it is therefore important to further investigate the roles of these factors in the transmission of HVs in wild reptiles.

Four pathogenic mechanisms are highly conserved among all HVs and include: (1) intranuclear replication and capsid assembly; (2) expression of DNA metabolic and synthetic enzymes; (3) cell destruction following the release of viral progenies; and (4) the maintenance of latency in natural host cells (Davison, 2014; Roizmann et al., 1992; Whitley, 1996). Generally, HV infections begin with viral entry, which is followed either by localisation or systemic spread (Davison, 2014). An experimental transmission study described the systemic dissemination of tortoise HVs (isolates HV 1976 and HV 4295/7R/95) (Origgi et al., 2004). Following experimental infection via intranasal and intramuscular routes, the HVs elicited clinical signs and were detected by PCR in tissue samples from the respiratory, digestive, and urogenital tracts, central nervous system (CNS), heart and spleen (78). Fibropapillomatosis (FP) and grey patch disease (GPD) are both associated with clinical signs that could be attributed to local destruction of infected cells due to replication and progeny release (Herbst et al., 1999; Monezi et al., 2016; Page-Karjian et al., 2017; Rebell et al., 1975; Work et al., 2017). Evidentially, two studies demonstrated the local replication of ChHV-5 by detecting certain biomarkers (eosinophilic intranuclear inclusions, F-VP26, DNA, and mRNA transcripts) within fibropapillomatous lesions (Kang et al., 2008; Work et al., 2015).

Unfortunately, the specific mechanisms involved in host cell invasion, immune evasion, localisation and spread of reptilian HVs have not been fully elucidated. However, recent molecular studies have provided insights into some virulence factors associated with reptilian HVs (Ackermann et al., 2012; Gandar et al., 2015; Origgi et al., 2015). Briefly, glycoproteins

B (gB), gC, gD, gH, gK gL, gM, and gN have been hypothesised to function in host cell attachment and entry (Ackermann et al., 2012; Gandar et al., 2015; Origgi et al., 2015). gB and gC are capable of binding to heparan sulphate proteoglycans that are present on the surface of many cells, thus aiding viral adsorption and penetration into different cell types (Engels & Ackermann, 1996; Gandar et al., 2015). The interactions of gB, gD, gH, gK, gL, gM, and gN have also been postulated to mediate membrane fusion and viral entry into the cell (Engels & Ackermann, 1996; Gandar et al., 2015; Origgi et al., 2015). Glycoproteins B, E, H, and L are involved in viral cell to cell spread, which could occur through intercellular bridges or intra-axonal transport, thus circumventing humoral immune responses (Gandar et al., 2015; Origgi et al., 2015). Similarly, the gC can bind to the third complement component (C3b) to block the alternative pathway complement activation (Gandar et al., 2015; Huemer et al., 1993). The gE and gI in HSV-1 inhibit the normal function of antibodies by building up a complex that acts as an Fc-receptor (Jacobs, 1994). However, the immunosuppression mechanism of the gE homologue in reptilian HVs is not yet clear. Finally, the F-M04 and F-sial proteins were recently identified in ChHV-5 and thought to play a role in FP pathogenesis; however, the specific mechanisms involved are not yet understood (Ackermann et al., 2012). Future research should consolidate characterisation of reptilian HVs in order to increase the understanding of host-pathogen interactions and improve clinical interventions.

2.3.7. Clinical and Pathological Signs

Herpesvirus infections have been described in reptiles with a range of clinical manifestations (Ariel, 2011). To provide an overview, the clinical signs and the gross and histological lesions associated with reptilian HVs are summarised in Table 2.2. Some of the more detailed descriptions are from sea turtles, tortoises, and crocodiles. Grey patch disease and FP, characterised by coalescing greyish papular skin lesions (spreading patches) and branching papillary tumours (Figure 2.6), respectively, have been reported in sea turtles (Aguirre et al.,

1999; Cárdenas et al., 2019; Rebell et al., 1975; Stacy et al., 2008). Lung-eye-trachea disease (LETD) with a clinical course of 2–3 weeks has been seen in green sea turtles (Jacobson et al., 1986). Lung-eye-trachea diseased turtles often present with pneumonia, stridor and caseation of the eyes, oropharynx and trachea (Jacobson et al., 1986). In non-marine turtles, HV infections are associated with hepatic necrosis, and proliferative and/or ulcerative lesions of the skin and shell (Figure 2.7) (Cowan et al., 2015; Ossiboff et al., 2015; Široký et al., 2018; Yonkers et al., 2015). Infections in tortoises result in ulcerative to diphtheroid-necrotizing stomatitis, conjunctivitis, glossitis, rhinitis, dyspnoea, liver disease and neurological disease and could be accompanied by anorexia, regurgitation, neck oedema, lethargy, and death (Braune et al., 1989; Hervás et al., 2002; Hunt, 2006; McArthur et al., 2002). Papillomas, stomatitis, and hepatitis are commonly described in lizards infected with HV (Goe et al., 2016; Hughes-Hanks et al., 2010; Literak et al., 2010; Raynaud & Adrian, 1976; Wellehan et al., 2005; Wellehan et al., 2004). Recently, five green snakes (*Opheodrys vernalis*) housed together presented with oropharyngeal squamous cell carcinoma and molecular analysis confirmed the presence of a novel Opheodrys herpesvirus-1 (Alphaherpesvirinae) (Lovstad et al., 2019). In another study, a lymphoid follicular cloacal inflammation in juvenile alligators was initially associated with tortoise HV. However, the HV (Genbank accession AY913769.1) was later determined to be a likely laboratory contamination and the actual causative agent is still unknown (Govett et al., 2005). Similarly, Hyndman et al. identified three novel HVs associated with conjunctivitis and/or pharyngitis (CP), systemic lymphoid proliferation with non-suppurative encephalitis (SLPE), and lymphonodular skin lesions (LNS) in farmed saltwater crocodiles (*Crocodylus porosus*) and captive freshwater crocodiles (*Crocodylus johnstoni*) (Hyndman et al., 2015). Obviously, HVs can induce significant diseases in both captive and wild reptiles; therefore, there is a

need to develop rapid diagnostic tests that will aid disease surveillance and reporting in order to maintain safe biosecurity measures and reduce spread.

2.3.8. Epidemiology

Epidemiological studies of HVs in wild reptiles could be challenging due to a lack of sensitive diagnostics for the detection of unknown or novel species, especially in resource-limited regions. HV infections are commonly characterised by non-specific clinical signs in most reptiles, thus making diagnoses on the basis of clinical signs alone difficult. An exception to this is FP, in which the presence of cutaneous tumours gives an indication of the disease; hence, more FP-associated HV data have been reported in recent decades (Figure 2.2; Appendix 1: Supplementary Table 2.2). Even so, the complete disease impact on wild populations could be currently underestimated due to the underreporting of outbreaks, sampling bias and poor monitoring systems. For instance, there is a paucity of information for FP epidemiology at the pelagic phase of life in sea turtles as most studies are biased towards sampling nearshore juveniles and adult females at foraging grounds or nesting beaches. Nevertheless, wildlife workers and researchers who, despite numerous challenges, have provided considerable epidemiological data targeted at conservation efforts towards endangered species should be commended. An overview of some of the epidemiological information including the prevalence and demography of both wild and captive reptilian HVs is discussed in this section.

Table 2. 2. Clinical presentations, gross and histological lesions of reptilian HV species.

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
ChHV-1	• Green sea turtles (*Chelonia mydas*)	• Benign papular lesions on the neck and flippers. • Spreading greyish patches to large areas of the epidermal surface. • Death may occur.	• Benign Papules. • Spreading grey patches.	• Intranuclear inclusions found in epidermal keratinocytes.	(Haines, 1978; Haines et al., 1974; Rebell et al., 1975)
ChHV-2	• Pacific pond turtles *(Clemmys marmorata*)	• Lethargy. • Anorexia. • Muscular weakness. • Coma. • Subcutaneous haemorrhages. • Death.	• Hepatomegaly. • Pallor of kidney. • Subcutaneous Petechial and ecchymotic haemorrhages.	• Hepatic necrosis. • Intranuclear inclusion bodies. • Lymphocytic aggregation in liver, kidney, and spleen. • Moderately hyperplastic spleen.	(Frye et al., 1977)
ChHV-3	• Painted turtles (*Chrysemys picta*)	• Abscessation • Death	• Pulmonary edema. • Friable and greenish-brown liver. • Distended gall bladder. • Congested spleen. • Shell rot lesions on plastron.	• Foci of necrosis on the liver and infundibular septa. • Hepatocytes containing Eosinophilic intranuclear inclusions. • Granulocytic and mononuclear infiltrations.	(Cox et al., 1980)

ChHV-4	• Argentine tortoise (*Geochelone chilensis*)	• Acute death • Nasal discharge • Ocular discharge • Regurgitation • Anorexia • Lethargy • Necrotizing stomatitis	• Necrotizing lesions. • Serous atrophy of fat. • Pale liver.	• Diffuse area of necrosis in mucosal epithelium • Accumulation of necrotic cellular debris and fibrin. • Infiltration of Inflammatory cells. • Eosinophilic intranuclear inclusions within degenerating epithelial cells and other tissues. • Vacuolar degeneration of hepatocytes.	(Jacobson et al., 1985)
ChHV-5	• Green sea turtle (*Chelonia mydas*) • Loggerhead sea turtle (*Caretta caretta*) • Hawksbill turtle (*Eretmochelys imbricata*) • Leatherback turtle (*Dermochelys coriacea*) • Olive ridley sea turtle (*Lepidochelys olivacea*) • Kemp's ridley sea turtle	• Tumours on the inguine, tail, flippers, axillae, chin, neck, eyelids, corneas, carapace and plastron.	• Single to multiple raised cutaneous masses that are verrucous, smooth, sessile or pedunculated. • Ulcerated and necrotic large masses. • Pigmented cutaneous tumours. • Spherical, smooth, firm, white, or gelatinous and translucent nodules in the lungs, kidneys, liver, heart, and gastrointestinal tract.	• Papillary epidermal and dermal hyperplasia. • Orthokeratotic hyperkeratosis. • Hypertrophied epithelial cells overlying vascularized fibrous stroma. • Epithelial necrosis and multifocal areas of ballooning degeneration. • Lymphocytes and plasma cells infiltrations. • Melanophores within the masses. • Eosinophilic intranuclear inclusions.	(Herbst, 1994)

	(*Lepidochelys kempii*) • Flatback turtle (*Natator depressus*)				
ChHV-6	• Green sea turtles (*Chelonia mydas*)	• Gasping. • Buoyancy abnormalities. • Inability to dive. • Lethargy. • Caseous exudate covering the eyes, glottis and trachea. • Death.	• Emphysematous areas in the lungs. • Caseous exudate in the eyes, glottis and trachea. • Multifocal raised white nodules in the liver.	• Necrotic lesions in the glottis, tracheal and lungs. • Periglottal accumulations of necrotic cellular debris and fibrin. • Infiltrations of heterophils, lymphocytes and plasma cells in periglottal submucosa. • Periglottal and tracheal epithelial proliferative and/or squamous metaplastic changes. • Syncytial giant cells in tracheal mucosa and major airways of lungs. • Thickened interstitium. • Hypertrophic and hyperplastic cells with enlarged vacuolated nuclei lining the airways. • Amphophilic intranuclear inclusions.	(Jacobson et al., 1986)
TeHV-1	• Horsfield tortoises (*Testudo horsfieldii*) • Pancake tortoises	• Cervical extension. • Laboured breathing. • Respiratory murmur.	• Yellowish-white pseudomembrane in the mouth, pharynx and glottis. • Hepatomegaly and ecchymotic liver.	• Diffuse areas of degeneration and necrosis in tongue and pharynx and larynx. • Necrotic cellular debris and fibrin accumulation.	(Stöhr & Marschang, 2010; Une et al., 2000; Une et al., 1999)

	(*Malacochersus tornieri*)	• Oral and nasal discharge. • Reddish-white fibrinous coating of the tongue. • Death.	• Pseudomembrane formation in the stomach.	• Inflammatory cells infiltrations. • Eosinophilic or amphophilic inclusion bodies.	
TeHV-2	• Desert tortoises (*Gopherus agassizii*)	• Anorexia • Lethargy • Necrotizing stomatitis	• Oral plaques.	• Eosinophilic and amphophilic intranuclear inclusions in superficial epithelial cells. • Thick coagulum over the epithelial surfaces of the mouth, pharynx, and trachea. • Infiltration of heterophils, lymphocytes, plasma cells, and macrophages. • Granulation of oropharyngeal tissue following epithelial loss.	(Johnson et al., 2005)
TeHV-3	• Greek Tortoises (*Testudo graeca*). • Hermann's Tortoises (*Testudo hermanni*).	• Nasal and oral discharges. • Rhinitis. • Dyspnoea. • Conjunctivitis associated with blepharospasm. • Diphtheroid-necrotizing stomatitis. • Glossitis. • Pharyngitis.	• Stomatitis with yellowish oral plaques. • Rhinitis with foamy nasal discharge. • Conjunctivitis.	• Oesophageal hyperplasia. • Hyperplasia and hyperkeratosis in the oral mucosa. • Sloughing of the epithelial cells and multifocal erosion. • Glottal epithelial ulceration, hyperplasia and necrosis. • Heterophilic pustules. • Amphophilic intranuclear inclusion bodies.	(Heldstab & Bestetti, 1989; Marenzoni et al., 2018; Marschang et al., 2009; Origgi et al., 2004)

		• CNS involvement (Circling, head tilt, lethargy, circling, paralysis and incoordination). • Deaths.		• Heterophilic bronchitis and pneumonia. • Nuclear degeneration changes of the hepatocytes. • Ballooning degeneration renal and digestive organs.	
TeHV-4*	• Bowsprit tortoise (*Chersina angulata*) • Leopard tortoise (*Stigmochelys pardalis*)	• Asymptomatic in some cases. • Respiratory distress. • Increased salivation.	• No data	• No data	(Bicknese et al., 2010; Kolesnik et al., 2016)
LGRV	• Loggerhead sea turtles (*Caretta caretta*)	• Moribund state • Lethargy and quadriparesis • Emaciation • Abnormal gait. • Death	• Colon impaction. • Fibrinonecrotic colitis. • Linear ulcers around the base of the base of the phallus. • Multifocal ulcers along the mucocutaneous junction of the eyelids. • Circumferential ulcer around the entire mucocutaneous junction of the cloaca. • Ulcerative gastritis	• Epithelial hyperplasia. • Ballooning degeneration and syncytial cell formation within basal layers of the epithelium. • Intranuclear eosinophilic inclusion bodies. • Heterophilic inflammation.	(Stacy et al., 2008)

LOCV	• Loggerhead sea turtles (*Caretta caretta*)	• Moribund state. • Lethargy, bradycardia, hypoventilation, and aspiration pneumonia. • Death.	• Deep multifocal ulcers around the rostral aspect of the tongue. • Multifocal pale cutaneous plaques with erythematous borders on the ventral neck region. • Tenacious exudates covering some plaques. • Liver pallor.	• Eosinophilic intranuclear inclusion bodies. • Necrosis of the epithelium and extend into the underlying lingual collagen. • Heterophilic inflammation. • Epidermal hyperplasia. • Hyperkeratosis. • Intraepithelial pustules. • Multifocal serocellular crust. • Sloughed epithelial cells in the airways.	(Stacy et al., 2008)
EBHV-1*	• Blanding's turtles (*Emydoidea blandingii*)	Asymptomatic	• No data	• No data	(Lindemann et al., 2018)
GlyHV-1*	• Bog turtles (*Glyptemys muhlenbergii*)	Asymptomatic	• No data	• No data	(Ossiboff et al., 2015)
GlyHV-2*	• Wood turtles (*Glyptemys insculpta*)	Asymptomatic	• No data	• No data	(Ossiboff et al., 2015)
EmyHV-1	• Eastern river cooter (*Pseudemys concinna*) • Northern map turtle	• Weakness • Frothy nasal discharge • Acute death	• Dark red, wet, and heavy lungs. • Thickened, wet, and gelatinous cranial aspect of the lungs.	• Hepatic lipidosis • Intranuclear inclusion bodies. • Necrotic lesions in the lungs, liver and spleen.	(Jungwirth et al., 2014; Ossiboff et al., 2015)

	(*Graptemys geographica*) • Painted turtles (*Chrysemys picta*)		• Trace amount of watery fluid in the trachea. • Diffusely tan, and slightly rounded lobular edges of the liver.	• Granulocytic and lymphocytic interstitial infiltrations. • Acute congestion with multifocal haemorrhage. •	
EmyHV-2*	• Bog turtle (*Glyptemys muhlenbergii*) • Spotted turtles (*Clemmys guttata*)	Asymptomatic	• No data	• No data	(Ossiboff et al., 2015)
TerHV-1*	• Eastern box turtles (*Terrapene carolina carolina*)	• Lethargy • Dehydration • Dyspnoea • Moribund state with fibronecrotic stomatitis and cloacitis. • Conjunctivitis • Blepharoedema • Death	• No data	• Necrosis, ulceration and syncytia formation of the pharyngeal mucosal epithelium. • Eosinophilic to amphophilic intranuclear inclusions.	(Sim et al., 2015)
TerHV-2	• Eastern box turtles (*Terrapene carolina carolina*)	• Papillomatous skin lesions. • Anorexia.	• Cutaneous papillomas.	• Papillary hyperplasia of the epithelium. • Infiltrations of lymphocytes, plasma cells, and heterophils. • Epithelium covered by keratin and cell debris.	(Yonkers et al., 2015)

Pelomedusid HV-1*	• West African mud turtles (*Pelusios castaneus*)	• Asymptomatic	• No data	• No data	(Marschang et al., 2015)
IgHV-1	• Green iguana (*Iguana iguana*)	• Acute death.	• Thin body. • Generalized muscle wasting. • Loss of fat store.	• Hepatocellular necrosis. • Hepatic syncytia. • Eosinophilic intranuclear inclusions. • Stomach and intestinal ulceration and necrosis. • Acute renal tubular necrosis. • Splenic lymphoid atrophy or hypoplasia	(Clark & Karzon, 1972; Wilkinson et al., 2005; Zeigel & Clark, 1972)
IgHV-2	• San Esteban Chuckwalla (*Sauromalus varius*)	• Acute death.	• Haemorrhage in the lung. • Congestion of airway. • Pale liver.	• Diffuse hepatic necrosis, eosinophilic intranuclear inclusions. • Multifocal necrosis of the spleen. • Interstitial infiltrations of muscles by mononuclear leucocyte. • Fibrosis of muscle and gingiva.	(Wellehan et al., 2003)
Gerrhosaurid HV-1	• Sudan plated lizard (*Gerrhosaurus major*)	• Glossal stomatitis. • Severe dyspnoea.	• Raised and tanned periglottal tongue • Little body fat.	• Glottal trachea of granulocytic and lymphocytic inflammation with erosion of overlying epithelium.	(Wellehan et al., 2004)
Gerrhosaurid HV-2*	• Black-lined plated lizard (*Gerrhosaurus nigrolineatus*)	• Labial stomatitis.	• No data	• No data	(Wellehan et al., 2004)

Gerrhosaurid HV-3*	• Sudan plated lizard (*Gerrhosaurus major*)	• Chronic labial proliferative & ulcerative growth.	• No data	• No data	(Wellehan et al., 2004)
VHV-1	• Green tree monitor lizards (*Varanus prasinus*)	• Proliferative and Ulcerative stomatitis/gingivitis. • Squamous cell carcinomas.	• Small white chalky plaques in the coelomic membrane, thoracic musculature, liver, kidneys, heart, and joints fascial plane. • Gingival proliferation • Mucosal hyperplasia • Fibrinous exudate on the serosa of the gall bladder. • Oral villous-like proliferation with patches of focal erythema.	• Mucosal epithelial proliferation • Severe pulmonary, myocardial, hepatic and renal vascular thrombosis. • Sloughed tubular endothelial cells. • Gingival necrosis. • Hepatic lipidosis. • Hepatic and renal amyloidosis.	(Wellehan et al., 2005)
VHV-3	• Monitor Lizards (*Varanus spp.*)	• Acute death	• Yellow-tan or white viscous material and white, thick material in the intestine and distal colon respectively. • Multiple soft, white particles (2–3 mm) in intestinal tract. • Diffuse pale-brown liver with multiple flat, tan pinpoint foci on the capsular surface.	• Acute, multifocal, coagulative necrosis in the lamina propria of the small intestine. • Acute, multifocal hepatocellular coagulative necrosis. • Eosinophilic intranuclear inclusions in the small intestine and liver.	(Hughes-Hanks et al., 2010)

HeHV-1	• Gila monster (*Heloderma suspectum*)	• Intraoral mass. • Loss of weight.	• Gingival nodule. • Muscle atrophy.	• Anastomosing epithelial cords. • Proliferative gingival tissues. • Eosinophilic and birefringent material within mass.	(Goe et al., 2016)
Elapid HV-1	• Siamese cobra (*Naja naja kaouthia*)	• Thick tenacious venom (low grade venom).	• Enlarged venom gland. • Thick venom exudates.	• Venom glands are lined by degenerated epithelial cells. • Mononuclear cell infiltration of gland subepithelium. • Debris, degenerated cells and venom in the lumina of glands. • Intranuclear inclusions.	(Simpson et al., 1979)
Opheodrys HV-1	• Smooth green snakes (*Opheodrys vernalis*)	• Oropharyngeal squamous cell carcinoma.	• Pale tan, multinodular masses on oropharyngeal mucosa. • Brown friable accumulations on tumour surface.	• Distorted oropharyngeal mucosa and submucosa by epithelial neoplasm. • Islands of neoplastic epithelial cells containing keratin cores. • Anisocytosis and anisokaryosis of neoplastic epithelial cells. • Squamous differentiation, keratin pearls, prominent intercellular bridges. • Heterophilic inflammation and surface compact keratin layers.	(Lovstad et al., 2019)
CrHV-1	• Saltwater crocodiles (*Crocodylus porosus*)	• Conjunctivitis-pharyngitis (CP).	• Reddening and swelling of the conjunctivae of the eyelids and nictitating membrane.	• Epithelial Hyperplasia, erosion, or ulceration of the conjunctiva, pharynx and larynx with cellular infiltrations. • Lymphocyte, heterophil and macrophage infiltrations of	(Hyndman et al., 2015; Shilton et al., 2016)

			• Cornea opacity and rupture. • Fibrinocaseous conjunctival, lingual and oropharyngeal exudates.	cornea, iris, and conjunctival, pharyngeal and laryngeal epithelium.	
CrHV-2	• Saltwater crocodiles (*Crocodylus porosus*)	• Conjunctivitis-pharyngitis (CP). • Concurrent skin ulcers. • Systemic lymphoid proliferation and encephalitis (SLPE). • Lymphnodular skin (LNS).	**CP** • Gross lesions of CP syndrome as described above. **SLPE** • Poor body condition. • Splenomegaly. • Pulmonary edema. **LNS** • Pale, soft, raised, well-delineated foci on lateral abdominal scales with occasional ulcerated surface covered in caseous exudate • Pale pink soft glistening tissue between the epidermis and deep dermal collagen. • Enlarged tonsils with multinodular appearance.	**CP** • Histological lesions of CP syndrome as described above. **SLPE** • Lymphohistiocytic and macrophage infiltration of pulmonary septae, hepatic periportal regions, pancreatic interstitium, gastrointestinal submucosa, pericardium, epicardium, iris, wall of large blood vessels and brain. • Hyperplastic lymphocytic conjunctivitis. **LNS** • Expansion and displacement of collagen of the superficial and mid-dermis by intense multinodular mononuclear cell infiltrate. • Epithelial hyperplasia of the tonsils will lymphocytes and macrophage infiltrations.	(Hyndman et al., 2015; Shilton et al., 2016)

			• Discrete soft white foci in the subepithelial tissue of the conjunctiva. • Multinodular swelling of the cloacal mucosa. • Discrete white soft foci in the parenchyma of the myocardium, liver, or kidney.	• Dense lymphohistiocytic aggregates of myocardium, liver, or kidney.	
CrHV-3	Freshwater crocodiles (*Crocodylus johnstoni*)	• Systemic lymphoid proliferation.	• Gross lesions of SLPE described above.	• Histological lesions of SLPE described above	(Hyndman et al., 2015; Shilton et al., 2016)

ChHV: Chelonid herpesvirus; TeHV: Testudinid herpesvirus; LGRV: loggerhead genital-respiratory herpesvirus; LOCV: loggerhead orocutaneous herpesvirus; EBHV: Emydoidea herpesvirus; GlyHV: Glyptemys herpesvirus; EmyHV: Emydid herpesvirus; TerHV: Terrapene herpesvirus; IgHV: Iguanid herpesvirus; VHV: Varanid herpesvirus; HeHV: Helodermatid herpesvirus; CrHV: Crocodyline herpesvirus.

Asterisks (*): To the best of my knowledge, the gross or histological lesions of some novel viruses have either not been detected or were reported while this manuscript was being written.

Figure 2. 6. Fibropapillomatosis in green turtle (*Chelonia mydas*). Photo by Dr. Karina Jones.

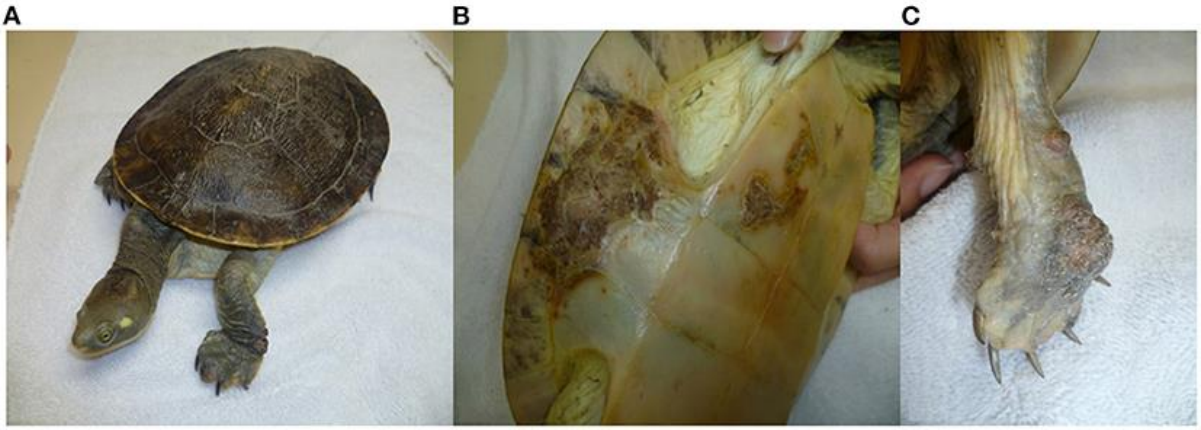


Figure 2. 7. Herpesvirus infection in freshwater turtle (*Emydura macquarii krefftii*) presented with proliferative and ulcerative lesions of the skin (A), proliferative and crusted lesions on the bridge of the shell (B), and proliferative lesion on the palmar aspect of the right forefoot (C). "Adapted from Herpesvirus in a captive Australian Krefft's river turtle (*Emydura macquarii krefftii*)" by Cowan et al. (Cowan et al., 2015). Copyright 2023 by John Wiley and Sons. Reprinted with permission.

Herpesviruses are linked to different diseases of marine turtles, including FP, LETD and GPD (Curry et al., 2000; Rebell et al., 1975). FP is a debilitating disease characterised by the development of tumours (dos Santos et al., 2010; Page-Karjian et al., 2014). Depending on the location of the tumours, FP can have detrimental effects (Cárdenas et al., 2019; Tagliolatto et al., 2016). On the basis of prevalence and distribution, Tagliolatto et al. reported a prevalence rate of 43% for FP in green turtles captured in a foraging area in south-eastern Brazil (Tagliolatto et al., 2016). Adnyana et al. recorded 22% overall prevalence in green turtles in Indonesia and also observed that the prevalence rate of FP was higher among turtles from waters adjacent to densely populated regions compared to those collected from waters remote from urbanised regions of Indonesia (Adnyana et al., 1997). These findings indicate that the epidemiology of FP in marine turtles vary between geographical regions and may be linked to anthropogenic activity. This theory is supported by the findings in another study, which attributed the variation of FP prevalence to environmental cofactors that vary among local habitats (Ene et al., 2005). A study associated the geographical distribution of FP with the genomic variation of HVs in marine turtles and observed four forms of the virus corresponding to Atlantic Ocean, west Pacific, mid-Pacific, and east Pacific (Greenblatt et al., 2005). A similar study conducted in Australian waters identified different genotypes along the east coast of Queensland. Such differences in strains may also effectuate different levels of pathogenicity between strains (Ariel et al., 2017; Jones et al., 2020), and account for

variation in reported prevalence in different regions. Also, given that the immune system of reptiles is dependent on temperature (Rios & Zimmerman, 2015; Zimmerman et al., 2010), the variation in the prevalence rates of reptilian HVs across regions could be associated with differences in regional climate types. Comprehensive reviews of the epidemiology of FP in marine turtles have been documented elsewhere (Herbst et al., 2004; Herbst, 1994; Herbst & Klein, 1995; Jones, 2004; Jones et al., 2016). The host immune status influences the clinical course of a disease, as immuno-deficient populations are more likely to succumb to disease outbreaks. Serosurveys have been conducted to determine the immune status of populations and to provide evidence of past and ongoing HV infections (Willimann, 2018; Winter et al., 2018). Seroepidemiological studies in three localities in Florida revealed high anti-ChHV seroprevalences (up to 100%) in both FP and non-FP sea turtles (Herbst et al., 1998; Herbst et al., 2008). Contrastingly, seropositivity to ChHV-5 was dependent on the tumour status in turtles from Hawaii (Work et al., 2020). This variation was mainly attributed to differences in the pathogenicity of ChHV-5 subtypes from the two regions (Work et al., 2020). In another report, an epizootic of LETD in confined juvenile green sea turtles resulted in 8 to 38% mortality, thus posing significant conservation and management concerns (Coberley, Herbst, Brown, et al., 2001; Jacobson et al., 1986). The LETD impact on free ranging sea turtles has not been investigated; however, seroprevalence rates of 13% and 22% were reported in two studies, respectively (Coberley et al., 2001; Coberley et al., 2001).

Similarly, HV infections are causing increasingly significant concerns in non-marine chelonians (Adamovicz et al., 2018; Lindemann et al., 2018). Herpesviruses have been implicated as the cause of severe clinical signs and acute death in terrestrial and freshwater turtles (Table 2.2) (Cowan et al., 2015; Jacobson et al., 1982; Jungwirth et al., 2014; Ossiboff et al., 2015; Sim et al., 2015; Široký et al., 2018). Although, HVs have been associated with latent infections in their natural hosts, infections in young, immunosuppressed or non-adapted

hosts could result in the development of significant diseases (Divers & Stahl, 2018). Therefore, monitoring the disease impact on both wild and captive endangered species has become pertinent. In an epidemiological study conducted in Tennessee and Illinois, USA, 128 of 409 free-ranging eastern box turtles (*Terrapene carolina carolina*) tested positive for TeHV-1 using TaqMan quantitative PCR, and the detection rate varied widely between seasons (Kane et al., 2017). Another study reported 48.3% prevalence of HV infections in endangered populations of bog (*Glyptemys muhlenbergii*), wood (*G. insculpta*), and spotted (*Clemmys guttata*) turtles in the north-eastern United States (Ossiboff et al., 2015). Furthermore, tortoise HVs have been associated with high mortality and morbidity (Hunt, 2006; Kolesnik et al., 2016; Lange et al., 1989; Muro et al., 1998; Pettan-Brewer et al., 1996). Different HV species were identified to cause the death of a large number of pancake *(Malacochersus tornieri*), Horsfield (*Testudo horsfieldii*), Hermann's (*Testudo hermanni*), and Egyptian tortoises (*Testudo kleinmanni*) during spontaneous outbreaks in Japan, Italy and Germany, respectively (Marschang et al., 1997; Marschang et al., 2009; Une et al., 1999). Species dependent susceptibility to HV was reported in a tortoise colony in which *T. graeca* and *T. horsfieldii* appeared to be unaffected by the HV species that caused the death of other tortoises in the same colony (Hunt, 2006). A possible explanation could be that the causative HV species is well-adapted in these tortoises, and they could be transmitting the virus to naïve or non-adapted tortoises. Of the four tortoise HV species (TeHV1-4), TeHV-3 appears to be the most pathogenic and frequently described, causing lethal disease in different tortoise species (Marenzoni et al., 2018; Marschang, 2019; Marschang et al., 1997; Origgi & Rigoni, 2003; Origgi, 2012). In a recent assessment of the incidence of chelonian HVs in Europe, more than half (54%) of all the detected chelonian viruses were TeHV-3 (Kolesnik et al., 2017). Again, seroprevalence rates of 27% and 31% were reported for TeHV-1 and TeHV-3, respectively, in different populations of desert tortoises in California (Jacobson et al., 2012;

Johnson et al., 2006). Despite the significance of HV infections, I observed that the disease is still grossly under-studied in some countries (Table 2.1). Thus, insufficient data and underreporting have made it difficult to assess the geographical patterns of the HV epidemiology in non-marine chelonians and other reptiles.

Herpesviruses have also been described in various species of squamates and crocodilians (Goe et al., 2016; Govett et al., 2005; Literak et al., 2010; Lovstad et al., 2019; McCowan et al., 2004; Raynaud & Adrian, 1976; Simpson et al., 1979; Wellehan et al., 2005; Wellehan et al., 2004). A recent outbreak of a lethal HV infection in a private facility housing 127 snakes resulted in the death of all 71 horned vipers at the premises after a brief illness (Catoi et al., 2014). An earlier study also implicated HV in the death of some boa constrictors within the first year of life (Hauser et al., 1983). Herpesvirus-induced deaths have been reported in different species of lizards with case fatalities nearing 100% (Hughes-Hanks et al., 2010; Watson, 1993; Wilkinson et al., 2005). As stated earlier, HV infections in crocodiles are associated with CP, SLPE and LNS syndromes (Hyndman et al., 2015). Another study strongly linked HV infection to SLPE and CP syndromes in farmed Australian saltwater crocodiles, with the highest prevalence rates of 94 and 54%, respectively (Shilton et al., 2016). Crocodiles are intensively farmed for commercial purposes in Australia; therefore, the occurrence of HVs in crocodiles has both epidemiological and economic implications (Hyndman et al., 2015; Lott et al., 2018).

Finally, I extracted a total of 130 articles, of which 39% (51 articles) and 32% (41 articles) were studies that investigated HVs in marine turtles and tortoises, respectively. A total of 21 (16%) studies investigated HVs in freshwater turtles. HVs were least studied in lizards (8%; 11 articles), snakes (3%; 4 articles) and crocodiles (2%; 3 articles) (Figure 2.2). The scant studies of HVs in some reptilian species since the 1970s could be attributed to the unavailability of reagents or sensitive diagnostic assays required to investigate reptilian

diseases in remote areas or the lack of interest to investigate HVs in reptiles because of their relatively low socio-economic importance. Therefore, future efforts should be directed towards enhancing collaborations among government agencies, researchers and wildlife workers with a view to creating awareness, increasing access to reagents and sensitive assays, and ultimately conserving endangered reptiles.

2.3.9. Diagnosis

A timeline of reptilian HV diagnosis showed that traditional assays including histopathology, virus isolation (VI) and electron microscopy (EM) have been the mainstays in the diagnosis of reptilian HVs (Appendix 1: Supplementary Figure 2.2). Many studies have reported the use of these techniques since the 1970s for the investigation of reptilian HVs. A breakdown of the number of studies that have used these methods to detect reptilian HVs is shown in Figure 2.3. Molecular diagnosis of reptilian HVs started two decades ago and has been used increasingly since then (Figure 2.3; Appendix 1: Supplementary Figure 2.2). The advent of molecular diagnostic techniques has provided insight into the genetic characteristics and the phylogenetic relationship of most reptilian HVs. This section highlights some important characteristics of the various techniques used in the diagnosis of reptilian HVs.

Diagnosis of reptilian HVs is tentatively made on the basis of patient history, clinical signs, and gross and histological lesions (Ariel, 2011). However, this is not always the case, as host-adapted HVs can cause subclinical, mild or latent infections in their natural hosts, and the demonstration of intranuclear inclusions is not pathognomonic of reptilian HV infections (Divers & Mader, 2005). Intranuclear inclusions are frequently associated with other reptilian viruses including adenoviruses and papillomaviruses (Farkas & Gál, 2009; Hyndman & Shilton, 2011; Manire et al., 2008). Earlier researchers used EM to confirm the presence of reptilian HV infections by demonstrating the ultrastructure of the viral particles in fixed, cut

and stained sections of tissue samples (Harper et al., 1982; Jacobson et al., 1986; Jacobson et al., 1982; Lange et al., 1989; Raynaud & Adrian, 1976; Rebell et al., 1975; Simpson et al., 1979; Zeigel & Clark, 1972). More recently, EM has been used to confirm a necrotic hepatitis associated with HV infection in a tortoise with no clinical signs or lesions in the respiratory tract, oral cavity or other organs (Hervás et al., 2002). The need for high technical capacities and the high cost of electron microscopes limits the use of EM for epidemiological and diagnostic purposes especially in resource-limited areas. Despite these limitations, EM remains a powerful detection tool in most high-class virology laboratories.

Reptilian HVs have been isolated in cell culture and identified on the basis of their cytopathic effects (Clark & Karzon, 1972; Drury et al., 1999; Jacobson et al., 1986; Shilton et al., 2016). For instance, tortoise HVs were isolated from pharyngeal swabs, trachea, kidney, oesophagus, tongue, stomach, and intestine, and caused cytolysis and rounding of cells in terrapene heart cells (TH-1) (Marschang et al., 2001). In another study, detachment and foci of enlarged, rounded, refractile cells were produced following inoculation of tissue and swab supernatants in turtle heart cells (Marschang et al., 1997). ChHV-5, which historically has been resistant to replication in conventional cultures, produced de novo ballooning degeneration and eosinophilic intranuclear inclusion in plugs and organotypic skin cultures (Work et al., 2017). This observation implies that ChHV-5 remains latent in conventional cultures and requires replication of the turtle skin to grow in vitro (Work et al., 2017). Aside from the fact that CPE are not obtained for non-cytopathic viruses, cell culture is susceptible to both chemical and biological contaminations, which in turn affect its sensitivity and specificity. Also, diagnostic turnaround could be delayed for slow-growing viruses. Therefore, it should not be solely relied upon for the epidemiological investigations of HVs.

Following primary infections in reptiles, a strong non-specific (innate) immune response that includes lysozymes, leukocytes, natural antibodies (NAbs), antimicrobial peptides, and the

complement pathway, is quickly stimulated (Rios & Zimmerman, 2015; Zimmerman et al., 2010). No specific information is currently documented about adaptive cell mediated immunity to HV infections. Unlike mammals, in reptiles a less robust and slower humoral response (IgA, IgD, IgM, and IgY) is stimulated after the innate immune system is activated (Divers & Mader, 2005; Rios & Zimmerman, 2015). In tortoises, neutralising antibodies to HV infection were detectable in serum at least 4 weeks post-exposure (Origgi et al., 2001). These serum neutralising antibodies did not appear to confer immunity to reinfection or recrudescence (Origgi et al., 2004). Later seroconversion was observed (four months to one year) in green turtles (*Chelonia mydas*) that were experimentally infected with ChHV (Herbst et al., 1998; Herbst et al., 2008). Generally, the detection of anti-herpesvirus antibodies in a single sample could indicate previous or latent infection, while rising antibody titre in paired samples collected at least 6 weeks apart indicates active infection (Divers & Mader, 2005; Pasmans et al., 2008). Humoral antibodies are detected by serological assays such as serum neutralisation (SN) tests, ELISA, and immunoperoxidase (IP) assays (Herbst et al., 2008; Marschang & Schneider, 2007; Nie & Lu, 1999; Origgi et al., 2003). The SN test is considered the reference test for anti-herpesvirus antibody detection but has limitations such as a delayed turnaround, inherent assay arduity and the requirement for standard isolates (Origgi et al., 2001). ELISAs with high sensitivity and specificity have been developed and deployed in various seroepidemiological studies (Herbst et al., 1998; Herbst et al., 2008; Jacobson et al., 2012; Johnson et al., 2005; Nie & Lu, 1999; Origgi et al., 2001). However, a high degree of cross-reactivity that potentially affects assay specificity has been demonstrated among different tortoise HV isolates used as antigens in the ELISA (Jacobson et al., 2012; Origgi et al., 2001). Cross-reactivity could also occur in other reptilian HVs that share similar antigenic epitopes, giving false positive results and, thus, leading to unnecessary post-exposure interventions. Overall, serological diagnostic techniques are not useful for the early

diagnosis of reptilian HVs because of the delay in antibody response and the need for paired serum sample collection weeks apart with accurate timing. However, it can play an important role in retrospective studies and in the diagnosis of latent or asymptomatic patients.

Recent epidemiological studies have largely relied on molecular methods to identify potential genetic and environmental risk factors associated with reptilian HVs (Aplasca et al., 2019; Kane et al., 2017; Lawrance et al., 2018; Martel et al., 2009; Monezi et al., 2016; Sim et al., 2016). Species-specific PCR-based assays targeting specific gene segments of reptilian HVs have been developed and validated (Braun et al., 2014; Origgi et al., 2004). Lindeman et al. developed two quantitative PCR assays and recorded a detection limit as low as 1 viral copy per reaction using primers that targeted the EBHV-1 specific segment of DNA polymerase gene (UL30) (Lindemann et al., 2018). In another study, two TaqMan PCR assays developed to target the UL30 gene of TerHV-1 detected 10 viral copies per reaction (Kane et al., 2016). Conventional and heminested PCR assays using tortoise HV-specific primers have been developed with assay sensitivity of 103 and 101 DNA copies, respectively (Murakami et al., 2001). Alternatively, consensus PCR techniques developed by VanDevanter et al. have been employed for the molecular screening and novel detection of reptilian HV species (Bicknese et al., 2010; Lackovich et al., 1999; Lindemann et al., 2018; Lu, Wang, et al., 2000; Quackenbush et al., 1998; Salinas et al., 2011; Une et al., 2000; VanDevanter et al., 1996). Although the molecular assays for the diagnosis of reptilian HVs have demonstrated excellent performance, their use still presents a major challenge in remote areas due to high cost, complexity of instrumentation, aseptic technique requirement and the need for electricity to operate PCR machines.

In order to accurately estimate the magnitude and scope of a disease outbreak or occurrence, a case definition (that is, standard criteria for categorising diseases) would need to be established. One of the ways to achieve this is to make available rapid, sensitive and

affordable assays for confirming the presence of diseases. Rapid diagnostic immunoassays that use lateral flow or chromatographic strategies should be developed for the rapid diagnosis of reptilian HV infections in the field or point of care (POC) settings. This approach could overcome some of the above-mentioned diagnostic challenges, especially in low resource areas. However, the use of lateral flow immunoassays for viral detection in other species have been marred by low and varying sensitivities (Chen et al., 2020; Fedorko et al., 2006; Padgett et al., 2006; Yu et al., 2020). Sensitive molecular-based rapid assays are relatively expensive and yet to be employed for the diagnosis of reptilian HVs (Kohda et al., 2014; Lemieux et al., 2012; Shojaei et al., 2015; Voermans et al., 2016). I would propose an ultrasensitive format that combines PCR and immunoassay but then it can be argued that such a laboratory-based system is less rapid and has limited use in resource-limited laboratories (Loose et al., 2020; Wang et al., 2018). Rapid detection techniques such as Microfluidic chip immunoassay and Smartphone-based rapid telemonitoring system (SBRTS) are fast becoming powerful tools in the diagnosis of viral infections (Natesan et al., 2019; Phillips et al., 2019; Priye et al., 2017; Rodriguez-Moncayo et al., 2021; Sun et al., 2020; Yeo et al., 2016; Yeo et al., 2018; Yu et al., 2017; Zhu et al., 2020). Of particular interest, is the SBRTS that combines biosensor and smartphone functionalities to produce a rapid, sensitive and cheap detection system (Zhao et al., 2020). SBRTS has an average turnaround of 30 min, overcomes inherent problems associated with sample handling and preparation, and can remotely monitor and report data on disease occurrence, thus making it suitable for use in resource-limited countries (Natesan et al., 2019; Zhao et al., 2020). This assay if employed could tick all the boxes for the epidemiological investigation and reporting of reptilian HVs.

Herpesvirus diagnostic and epidemiological data should be interpreted with prudence because of the possible influence of coinfection variables that could cause the reactivation of seemingly latent HV infections. For instance, some studies have reported the detection of co-

pathogens in reptiles showing clinical signs, some of which are typical of HV infections (Adamovicz et al., 2018; Archer et al., 2017; Kolesnik et al., 2017; Salinas et al., 2011; Sim et al., 2016; Soares et al., 2004; Yonkers et al., 2015). These observations imply that the detection of HVs may not be the actual cause of the current disease, but because the immune system is compromised by other pathogens, the HVs recrudesce and become easier to detect. Both latency (decreases apparent prevalence and significance) and coinfections (increase apparent prevalence and may also falsely assign the clinical signs to the HV) will have an influence on the disease picture. Therefore, I recommend that biosecurity and conservation measures should include a multiplex pathogen detection model whenever possible in order to fully assess the health of reptilian populations.

2.3.10. Treatment, Prevention, and Control

Surgical excision, carbon dioxide (CO_2) laser surgery and cryosurgery are some of the commonly used therapeutic strategies for the management of HV-associated tumours (Cowan et al., 2015; Goe et al., 2016; Page-Karjian et al., 2014; Raiti, 2008; Schroeder & Witherington, 1994; Široký et al., 2018). High rates of recurrence and the risk of secondary bacterial infections have greatly reduced the efficacy of surgical excision (Page-Karjian et al., 2019). CO_2 laser surgery, which combines laser excision and ablation of tumours, has shown improved intraoperative and postoperative outcomes and is therefore the treatment of choice (Page-Karjian et al., 2014; Page-Karjian et al., 2019; Raiti, 2008). Non-surgical approaches including electrochemotherapy (ECT) and photodynamic therapy (PDT) with no known recurrence have recently been employed as alternatives in the treatment of FP (Brunner et al., 2014; Sellera et al., 2014).

Several authors have recommended the use of acyclovir complemented by fluid and antibiotic therapies for the effective treatment of tortoise HV infection (McArthur, 2000; Origgi &

Rigoni, 2003; Wright, 2008). Marschang et al. showed that acyclovir and ganciclovir effectively inhibited HV replication in vitro at a single dose or repeated daily dose of 25 or 50 µg/mL (Marschang et al., 1997). Similarly, the in vitro activities of acyclovir and ganciclovir were recently tested and shown to be effective against TeHV-3; however, the safety of these drugs is yet to be demonstrated in tortoises (Gandar et al., 2019). Based on the toxicity (on liver and kidney cells) and other biochemical data, this same study showed that eprociclovir is not suitable for use as anti-TeHV-3 in Hermann's tortoises and further in vivo assessment of other potential antiviral drugs was recommended (Gandar et al., 2019).

Recently, an autogenous vaccine therapy was proposed and used for the treatment of HV-associated papillomatosis in Williams' mud turtle (*Pelusios williamsi*) (Široký et al., 2018). The autogenous vaccine, which was aseptically prepared from excised fresh tissue induced substantial areas of necrosis of the papillomatous lesions, thus indicating the efficacy of the vaccine (Široký et al., 2018). Autogenous vaccines potentially contain relevant neoantigens that comparatively improve their efficacy (Sondak et al., 2006). However, their use could be limited by lack of sufficient tumours (in patients) needed to produce adequate vaccine doses. Also, no standard protocol exists for autogenous vaccine production and delivery, and patients' tumours may progress beyond the intervention stage before the vaccine becomes ready for delivery. Allogeneic vaccines on the other hand, can overcome some of the aforementioned challenges; however, they may lack the advantageous self-neoantigens (Sondak et al., 2006). In the past, an inactivated vaccine was evaluated against tortoise HV without success as no significant rise in antibody was detected in vaccinated tortoises after 369 days post vaccination (Marschang et al., 2001). DNA or mRNA-based vaccines have the capacity to induce both humoral and cellular immune responses and have shown promising outcomes against some animal and human diseases (Brisse et al., 2020; Deb et al., 2015; Jackson et al., 2020; Leal et al., 2018). Although vaccine research and development could be

costly, laborious and time-consuming, the nucleic acid vaccines hold the potential to significantly reduce HV-associated losses in captive collections and wild reptiles of conservation concerns.

Prevention is of utmost importance in the management of reptilian HV infections, since death may still occur following therapeutic interventions and recovered animals remain latent carriers (Origgi & Rigoni, 2003; Pasmans et al., 2008). Unfortunately, there are no established preventive or control measures for HV infections in wild populations of reptiles (Rao et al., 2020), which consequently presents a major conservation challenge. Environmental factors including degraded water quality caused by pollutants, increased water temperature, natural biotoxins, and high dietary arginine concentrations due to microalgae bloom have arguably been linked as cofactors in the development of FP in sea turtles (Aguirre et al., 1994; Arthur et al., 2008; dos Santos et al., 2010; Foley et al., 2005; Jones et al., 2016; Landsberg et al., 1999; Van Houtan et al., 2010; Van Houtan et al., 2014). Therefore, adopting conservation actions needed to regulate water and species management, as well as regulating human activities leading to climate change, would be sensible.

In captive reptiles, quarantine procedures and adequate testing of new acquisitions are strongly recommended (Lott et al., 2018; Martel et al., 2009; Pasmans et al., 2008). All previously infected or HV seropositive animals should be treated as latent carriers and potential shedders to naïve populations, as factors including stress, bad husbandry, illness or immunosuppression could reactivate the virus (Marenzoni et al., 2018; Martel et al., 2009; Pasmans et al., 2008). Generally, strict hygiene practises and adequate biosecurity should be followed in all facilities housing reptiles (Hilton-Taylor & Brackett, 2000; Murakami et al., 2001; Origgi et al., 2001; Stahl, 2001; Teifke et al., 2000).

2.3.11. Recent updates on reptilian herpesviruses

The first baselines of FP and ChHV-5 prevalence have been reported for West Africa green turtles (Monteiro et al., 2021). In this study, ChHV-5 was detected in 24 tumour biopsies and 27 normal skins from the 108 sampled turtles indicating viral persistence or multifactorial influence on the pathogenesis of FP associated ChHV-5. Another study reported a high occurrence of ChHV-5 in sea turtles with and without FP in Brazil and suggested that ChHV-5 requires environmental or immune-related cofactors to induce FP (Zamana et al., 2021). Based on new molecular and histological evidence, a papillomavirus (Chelonia mydas Papillomavirus 1) has been implicated as a cofactor or potential causative agent of FP (Mashkour et al., 2021). The emergence of ChHV-5 in new locations and rare host species have been reported, indicating increased viral spread and potential threat to the survival of the endangered sea turtles (James et al., 2021; Loganathan et al., 2021; Page-Karjian et al., 2021; Robben et al., 2023; Whitmore et al., 2021). Although the method of ChHV-5 transmission is still unclear, molecular evidence suggests that marine leeches could serve as vectors in the transmission of FP associated ChHV-5 in sea turtles (Rittenburg et al., 2021). ChHV-5 was identified as the cause of a conjunctival disease without FP in a captive loggerhead turtle (*Caretta caretta*), suggesting that ChHV-5 should be considered as a differential diagnosis in atypical ocular disease manifestations in sea turtles (Oriá et al., 2021). Overall, HV diagnostic and epidemiological data should be interpreted with care because of the possible influence of latency and coinfection variables that could respectively decrease apparent prevalence or significance and increase apparent prevalence or falsely assign the clinical signs to the HV. Therefore, a holistic approach should be employed whenever possible for the investigation and interpretation of HV data from both clinically healthy and diseased reptiles.

In other reptiles, novel HVs belonging to the subfamily *Alphaherpesvirinae* have been identified and associated with diseases. For instance, a novel emydoidea herpesvirus 2

(EBHV-2) was associated with squamous cell carcinoma in a free-ranging Blanding's turtle (*Emydoidea blandingii*) (Andersson et al., 2021). Chamaeleonid herpesvirus 1 (chamHV-1) was associated with cheilitis in two Panther Chameleons (*Furcifer pardalis*) (Hellebuyck et al., 2021). A terrapene alphaherpesvirus 3 (TerHV-3) was detected in three species of chelonians including a captive leopard tortoise (*Stigmochelys pardalis*), a steppe tortoise (*Agrionemys horsfieldii*) and two free-living, three-toed box turtles (*Terrapene mexicana triunguis*) in USA. Clinical signs including respiratory tract disease, mucopurulent nasal discharge, lethargy, and death were observed, however, the link between these clinical signs and TerHV3 was not established in this study (Winter et al., 2022). Similarly, previously identified HVs including TeHV-1 and EmyHV-1 were recently identified as the cause of severe disease outbreaks in Horsfield's tortoises (*Testudo horsfieldii*) and western pond turtles (*Actinemys marmorata*) respectively (Schüler et al., 2021; Sim et al., 2021; Vetere et al., 2021). Several studies have conducted health assessment to determine the presence HVs in healthy populations of reptiles, and this has led to the detection of potentially pathogenic novel HVs in some cases (Calle et al., 2021; Marschang et al., 2021; Nieto-Claudin et al., 2022; Schönbächler et al., 2022; Vorbach et al., 2022; Willis et al., 2022). Herpesvirus infections do not always produce overt clinical signs, and this could be due to viral latency (latent infection) or viral suppression (asymptomatic infection) by the host immune system (which may get overwhelmed with time) (Grinde, 2013; Weidner-Glunde et al., 2020). Factors such as stress, co-infections, and immuno-suppression can exacerbate clinical conditions or reactivate the virus to cause serious illness in apparently healthy hosts (Sehrawat et al., 2018). Nonetheless, the increase in reporting (32 articles) over the past two years (Figure 2.8) supports the notion that HVs have continued to threaten the health and wellbeing of wild and captive reptiles with serious economic and conservation implications,

therefore routine health assessment and adequate control measures should be well implemented.

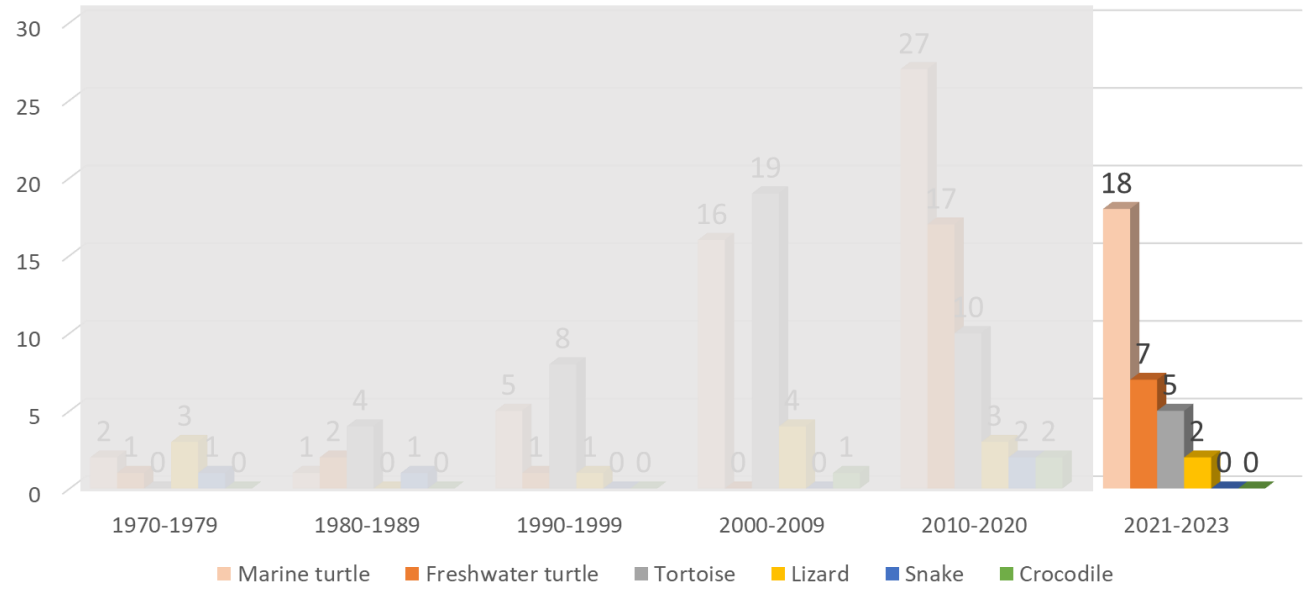


Figure 2. 8. Recent update on the number of studies on reptilian HVs from 2021 to 2023. The earlier reported studies (1970-2020) were shaded out.

2.4. Part II: Viruses Infecting Australian Marsupials: A Scoping Review

2.4.1. Introduction

Marsupials (Metatherian) are a group of mammals belonging to the infraclass Marsupialia and comprise over 300 extant species that are mostly found on the Australian continent (Larramendy & Liwszyc, 2022; Macri, 2018; Meredith et al., 2008). Marsupials constitute more than half of Australia's land mammals; they live in a wide range of habitats including dry scrub, desert, alpine and rainforest (Black et al., 2012; Kirsch & Calaby, 1977). Marsupials are believed to have evolved, together with placental mammals (Eutherians) from Therian mammals; and diverged from the Eutherians about 125 to 148 million years ago (Cheyne & Dennis, 2007; Menzies et al., 2011; Temple-Smith et al., 2018; Williamson et al., 2014). Marsupials migrated into Australia from North America in the late cretaceous or early tertiary period through Antarctica (Goin et al., 2016; Cheyne & Dennis, 2007; Nilsson et al., 2010). The independent evolution of Australian marsupials began when the Australian continent broke off from Antarctica and became separated from other land masses (Fooden, 1972; Cheyne & Dennis, 2007). Australian marsupials are grouped into four orders (Dasyuromorphia, Peramelemorphia, Diprotodontia and Notoryctemorphia) and can be further categorised into three subgroups based on their feeding habits and these include the carnivorous Dasyuromorphs (quolls, the Tasmanian devil, Tasmanian tiger, numbats, dunnarts, and antechinus) and Notoryctemorphs (marsupial moles), the omnivorous Peramelemorphs (bilbies and bandicoots) and Diprotodonts (burramyids, acrobatids and petaurids), and the herbivorous Diprotodonts (kangaroos, wallabies, possums, koalas, wombats) (Black et al., 2012; Gallus et al., 2015). Marsupials serve as pollinators and seed

distributors; help control pest by eating insects and vermin and contribute to healthy ecosystems (Fleming et al., 2014; Katumo et al., 2022; Valentine et al., 2017).

The conservation of some marsupial species in Australia is under threat and this is due to several factors including habitat loss, inappropriate fire regimes, introduced species, overexploitation, and diseases (Evans et al., 2011; Kearney et al., 2019). Historically, these factors have led to the extinction of some Australian marsupial species such as worongs, oolacuntas, desert bandicoots, lesser bilbies (yallaras), pig-footed bandicoots and thylacine (Black et al., 2012; Johnson, 2006; Short & Smith, 1994). Currently, species such as the Kangaroo Island dunnart, mountain pygmy-possum, Gilbert's potoroo, northern hairy-nosed wombat and woylie are considered endangered or critically endangered (IUCN, 2023). Thus, it has become necessary to consider and provide insights into specific potential factors of conservation concerns, which in turn, would aid policy makers and other stakeholders to formulate and implement effective conservation plans and preventive strategies.

Marsupial viruses are grossly understudied compared to their placental mammal counterparts, even though they pose severe threats to endangered marsupials (Harding et al., 2021). Other non-viral diseases including chlamydiosis in koalas, mange in wombats as well as the tumour disease in Tasmanian devil (devil facial tumour disease) are well reported to have population level effects but little is known about the epidemiology of viruses infecting Australian marsupials (Broughton, 2018; Hamede et al., 2015; Harding et al., 2021; Woods et al., 2018). The lack of epidemiological data has limited the understanding of the impacts of viruses on the health of Australian marsupials. Therefore, the aim of this study was to provide an overview of existing evidence on the occurrence and clinical significance of viruses that infect marsupials in Australia.

2.4.2. Methods

This review is reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses Extension for Scoping Reviews (PRISMA-ScR) and conducted using the Arksey and O'Malley framework (Tricco et al., 2018). This means that results are reported separately from discussion. A systematic literature search was performed (up to January 2023) in Medline (Ovid), PubMed, SCOPUS, and Web of Science. The keywords used and search strategies are available in the supplementary document (Appendix 1: Supplementary Table 2.3). The identified articles were screened to remove duplicates and non-relevant articles. I included peer reviewed, English-written and original research articles. Non-original research, guidelines and reviews were excluded. The information extracted from eligible articles included first author's name, publication year, study period, study location, sample type, diagnostic tests, biomarkers, as well as the key epidemiological data including viral species, observed clinical signs, marsupial host, number of animals examined, and numbers infected. Data analysis was done following the classification of the viral species into their respective family. Wherever possible, statistical analysis using odds ratio (OR) was used to determine an association of viral detection with clinical symptoms, and $P < 0.05$ was considered significant (MedCalc Software Ltd; Thrusfield, 2007).

2.4.3. Results

A total of 387 articles were identified, of which 192 were removed as duplicate articles, 31 were excluded as irrelevant after title and abstract screening, and six had non-retrievable full text (Figure 2.9). Of the remaining 158 full text articles assessed for eligibility, 87 articles were included in this review (Figure 2.9).

The included articles comprise virological and serological studies on marsupial viruses in Australia from 1963 to 2022. Except where otherwise stated, all virological detections

reported in this review were done using any one of the virus-based assays including histopathology (viral inclusion bodies), viral isolation (viral isolates), electron microscopy (viral particles), immunohistochemistry (viral antigen), PCR based assays (viral nucleic acids), and next generation sequencing (viral nucleic acids/genomes). Whereas serological screenings were conducted by antibody-based assays such as viral neutralization, haemagglutination-inhibition, and enzyme linked immunosorbent assays. Viruses in the family *Retroviridae* appear to be the most investigated (n = 30), and this was followed by *Herpesviridae* (n = 23) and *Togaviridae* (n = 11) (Figure 2.10). Several other viruses are reported in the literature during this period at varying frequencies including papillomavirus (n = 7), picornavirus (n = 6), flavivirus (n = 5), poxvirus (n = 5), sedoreovirus (n = 4), peribunyavirus (n = 3), and arterivirus (n =2), and one report for each of astrovirus, circovirus, parvovirus, picobirnaviruses, polyomavirus and spinareoviruses (Figure 2.10). An overview of the number of viral investigations in captive and wild marsupials is provided in a Supplementary Figure (Appendix 1: Supplementary Figure 2.3). The number of studies of marsupial viruses by different states in Australia (Figure 2.11) showed that the retroviruses were the most investigated in Queensland (Qld), New South Wales (NSW), Victoria (Vic) and South Australia (SA); while herpesviruses were the most investigated in Tasmania (Tas), Western Australia (WA) and Vic (Figure 2.11). The geographical distribution of different marsupial species in Australia may be a determining factor on the type and number of viral investigations across the different states, however, this factor was not considered in this study. I analysed the sero- and virological occurrences, and the clinical significance of marsupial viruses in a family-wise manner as presented below (Figure 2.10 and Figure 2.11).

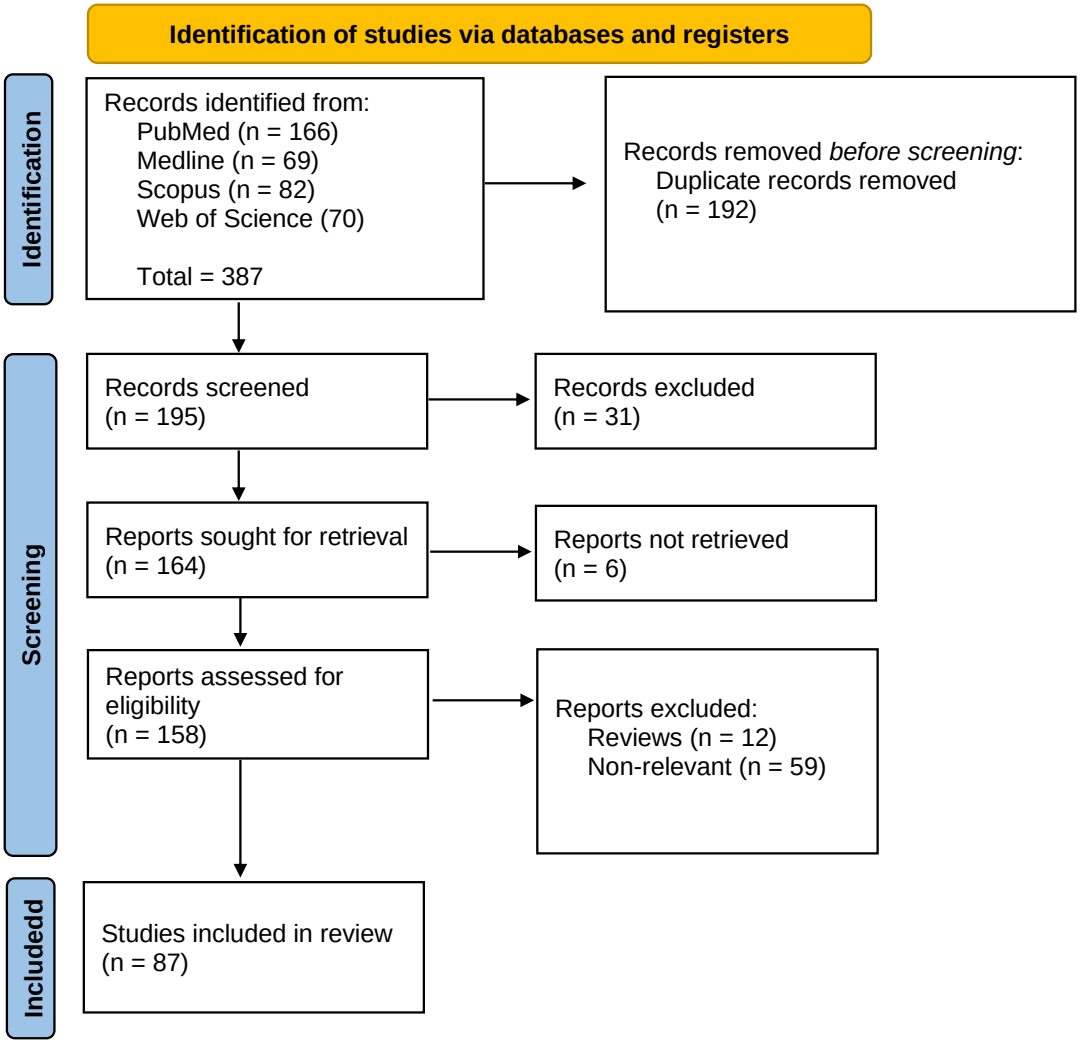


Figure 2. 9. PRISMA flow diagram for the scoping review process.

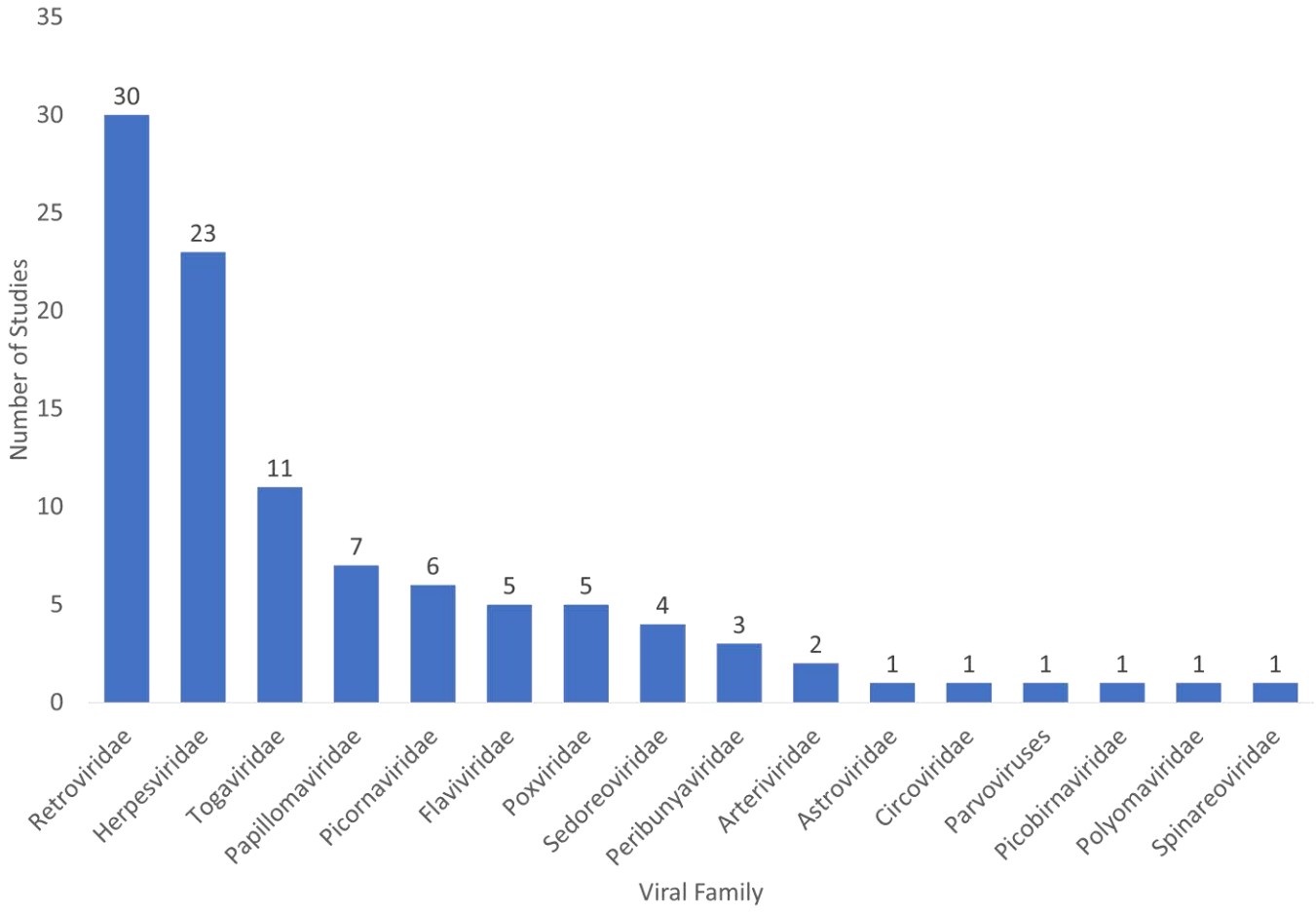


Figure 2. 10. Number of studies published in peer review journals on marsupial viruses in Australia from 1963 to 2023**.**

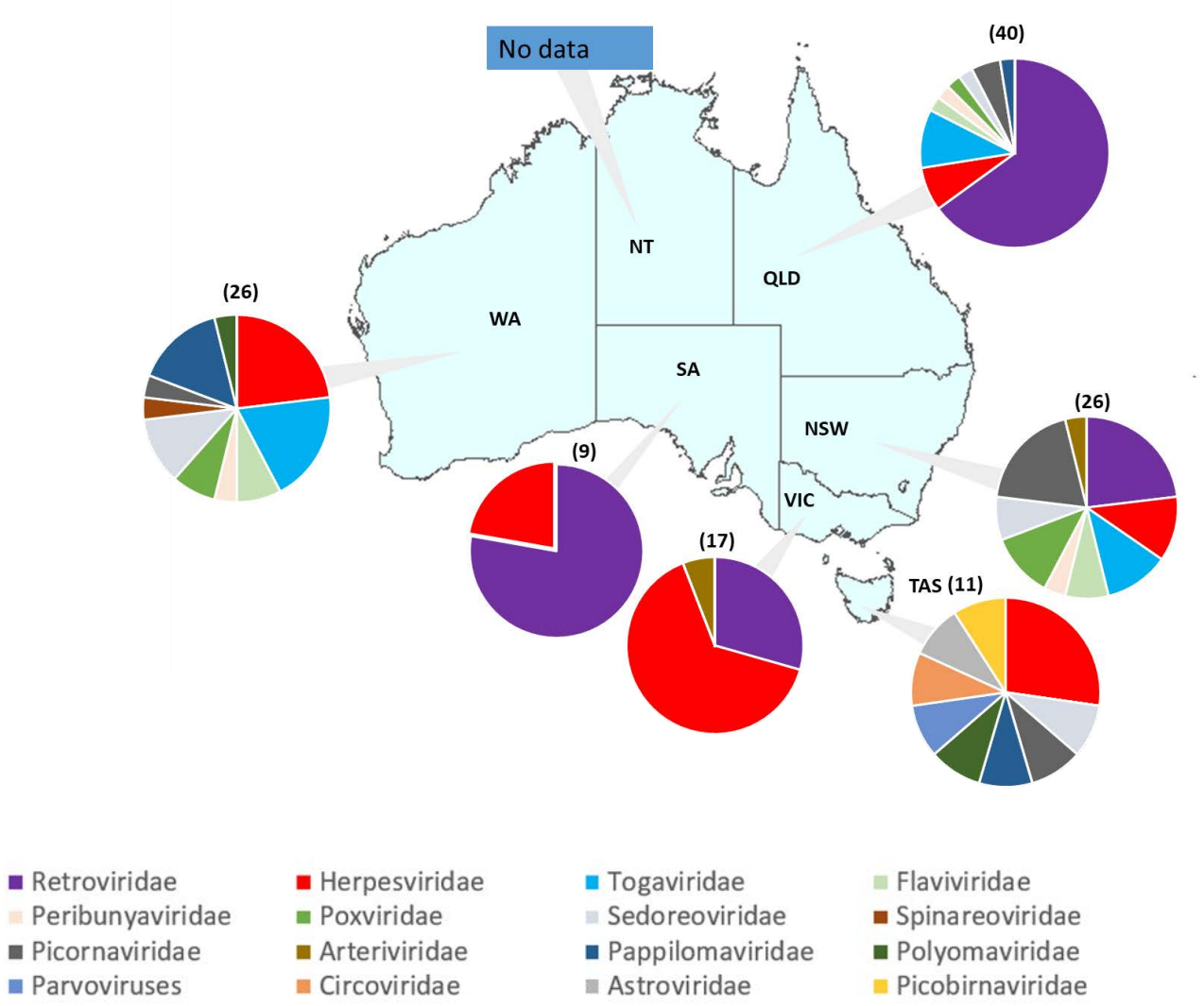


Figure 2. 11. Number of studies on marsupial viruses by states in Australia. The numbers in parenthesis represent the total number of investigations for each state.

Retroviridae

The retroviral species, koala retrovirus (KoRV) infect koalas in Australia. This virus was first identified in 1988 and the genome was fully sequenced in 2000 (Hanger et al., 2000). So far, nine subtypes of KoRV have been identified (KoRV-A to KoRV-I) (Quigley et al., 2021). KoRV is thought to be associated with immunosuppression and neoplasia; however, there is little or no evidence to strongly support these associations in the current literature (Blyton et al., 2022; Sarker et al., 2020; Sarker et al., 2019; Sarker et al., 2020; Tarlinton et al., 2005).

Virological screening of various biological samples showed that KoRV has been detected in 2894 koalas of the 4018 tested using molecular-based screening methods (2894/4018; 72% virological prevalence) (Ávila-Arcos et al., 2013; Blyton et al., 2022; Blyton et al., 2022; Chappell et al., 2017; Fabijan et al., 2019; Fiebig et al., 2015; Hanger et al., 2000; Hobbs et al., 2017; Hobbs et al., 2014; Joyce et al., 2022; Joyce et al., 2021; Legione et al., 2017; McEwen et al., 2021; Olagoke et al., 2019; Quigley et al., 2018; Quigley et al., 2019; Quigley et al., 2021; Robbins et al., 2020; Sarker et al., 2020; Sarker et al., 2019; Sarker et al., 2020; Simmons et al., 2012; Tarlinton et al., 2005; Tarlinton et al., 2022; Tarlinton et al., 2006; Waugh et al., 2017; Zheng et al., 2020). A total of 232 koalas were positive for KoRV out of the 244 serologically screened (232/244; 95% seroprevalence) (Fiebig et al., 2015; Olagoke et al., 2019). Of these, 62 (62/4262) had different neoplastic conditions including lymphoma, leukemia osteochondroma, mesothelioma, mammary tumour, rhabdomyoma, fibrosarcoma, anaplastic carcinoma, and lymphosarcoma (Appendix 1: Supplementary Table 2.3). I analysed these combined results with odds ratio (as a larger sample set rather than individual population) to determine an association between retrovirus infection and neoplasia. Overall, there was a statistically significant association between retrovirus infection and neoplasia in marsupials (P = 0.0020; Appendix 1: Supplementary Table 2.4). Retroviruses have been detected in four brushtail possums (4/4), one ringtail possums (1/1) and two sugar gliders (2/2) with no clinical signs (Simmons et al., 2014). Other marsupials including common wombat (0/5), Tasmanian devil (0/1), red necked wallaby (0/4), and stripe faced dunnart (0/5) were negative when tested for retroviruses (Simmons et al., 2014).

Herpesviridae

Several species of herpesviruses (HVs) have been found to infect marsupials in Australia including dasyurid herpesvirus 1-2, Lumholtz's Tree-kangaroo herpesvirus, macropodid herpesvirus 1-6, peramelid herpesvirus 1-2, phascolarctid herpesvirus 1-3, potoroid

herpesvirus 1, and vombatid herpesvirus 1-3. These viruses have been associated with clinical diseases that ranged from mild to severe, and include dyspnoea, wasting, anorexia, urogenital conditions, conjunctivitis, oculo-nasal discharges, and deaths (Amery-Gale et al., 2014; Bowater et al., 2022; Callinan & Kefford, 1981; Shima et al., 2020; Vaz et al., 2011; Vaz et al., 2019a; Vaz et al., 2019b; Wilcox et al., 2011; Wilks et al., 1981). HVs have a wide host range and have been detected at different virological and serological rates (Table 2.3). Overall, HVs were virologically and serologically detected in 696 (696/2937) and 191 marsupials (191/1233) respectively. Of all the marsupials tested, 64 (64/4170) exhibited one of the clinical signs listed in Table 2.3. Odds ratio was not considered for HVs due to the non-specific signs associated with HV infections coupled with the effects of latency and coinfection variables.

Table 2. 3. A summary of findings from virological and serological testing of herpesviruses in Australian marsupials.

Viral species	Marsupials	Method	No. Infected	No. tested	Observed clinical signs	References
Virological testing						
Alphaherpesvirinae						
Macropodid herpesvirus 1	Parma wallabies	Hist, EM, VI	16	20	Respiratory rales, conjunctivitis, incoordination, moribundity and death	(Finnie et al., 1976)
Macropodid herpesvirus 2	Woylies	Hist, EM, VI	8	8	Death over five days	(Dickson et al., 1980; Johnson et al., 1985)
	Rufous rat kangaroos	Hist, EM, VI	11	11	Death over five-day period	(Dickson et al., 1980; Johnson et al., 1985)
	Dorcopsis wallabies	Hist, EM, VI	18	22	Moribundity followed by death within 24 hrs	(Callinan & Kefford, 1981; Johnson et al., 1985;

						Wilks et al., 1981)
	Quokkas	Hist, EM, VI	2	2	Conjunctivitis, vesicles were observed on the mucosa of the mouth and cloaca and death within 24hrs of observing clinical signs.	(Callinan & Kefford, 1981; Johnson et al., 1985; Stalder et al., 2015; Wilks et al., 1981)
Macropodid herpesvirus 4	Lumholtz's Tree-kangaroos	Hist, PCR	1	20	Acute respiratory signs, oculo-nasal discharge and death	(Shima et al., 2020)
	Eastern grey kangaroos	VI, PCR	6	97	None	(Stalder et al., 2015; Vaz et al., 2013)
Phascolarctid herpesvirus 3	Koalas	Hist, PCR	1	1	Lethargy, weakness, anorexia and severe dyspnoea	(Bowater et al., 2022)
Vombatid herpesvirus 3	Common wombats	PCR	3	33	None	(Stalder et al., 2015)
Gammaherpesvirinae						
Lumholtz's tree-kangaroo herpesvirus	Lumholtz's tree-kangaroos	Hist, PCR	17	20	Lethargy, tachypnoea, dyspnoea in one positive kangaroo	(Shima et al., 2020)
Dasyurid herpesvirus 1	Yellow-footed antechinus and agile antechinuses	PCR	2	2	Weakness, poor body condition, death of agile antechinus	(Amery-Gale et al., 2014)
Dasyurid herpesvirus 2	Tasmanian devils	PCR	17	50	None	(Stalder et al., 2015)
Macropodid herpesvirus 3	Eastern grey kangaroos	VI, PCR	20	97	Bilateral serous ocular and nasal discharges	(Stalder et al., 2015; Wilcox et al., 2011)
Macropodid herpesvirus 5	Swamp wallabies	PCR	4	15	None	(Stalder et al., 2015)
Macropodid herpesvirus 6	Quokkas	PCR	26	179	None	(Martínez-Pérez et al., 2021; Martínez-Pérez et al., 2020)

Peramelid herpesvirus 1	Southern brown bandicoots	PCR	1	11	None	(Stalder et al., 2015)
Peramelid herpesvirus 2	Northern Brown Bandicoots	PCR	18	35	None	(Langhorne et al., 2021)
Phascolarctid herpesvirus 1	Koalas	VI, PCR, NGS	445	1893	Dermatitis, nephritis, cystitis, weakness, conjunctivitis, urogenital abnormality, and wet bottom	(Kasimov et al., 2020; Stalder et al., 2015; Vaz et al., 2012; Vaz et al., 2011; Vaz et al., 2019a; Vaz et al., 2019b)
Phascolarctid herpesvirus 2	Koalas	PCR	63	266	None	(Kasimov et al., 2020; Stalder et al., 2015)
Potoroid herpesvirus 1	Woylies	PCR	3	45	None	(Skogvold et al., 2017)
Vombatid herpesvirus 1	Common wombats	VI, PCR, NGS	6	34	None	(Stalder et al., 2015; Vaz et al., 2019b)
Vombatid herpesvirus 2	Common wombats	PCR	7	33	None	(Stalder et al., 2015)
Unknown	Western grey kangaroo	Hist	1	1	Dehydration, dyspnoea, death	(Callinan & Kefford, 1981)
	Tammar wallabies	PCR*	0	8	None	(Stalder et al., 2015)
	Yellow-footed rock wallabies	PCR	0	3	None	(Stalder et al., 2015)
	Red-necked wallaby	PCR	0	1	None	(Stalder et al., 2015)
	Long-nosed potoroos	PCR	0	9	None	(Stalder et al., 2015)
	Eastern quolls	PCR	0	2	None	(Stalder et al., 2015)
	Eastern barred bandicoots	PCR	0	3	None	(Stalder et al., 2015)

	Common brushtail possums	PCR	0	2	None	(Stalder et al., 2015)
	Common ringtail possums	PCR	0	1	None	(Stalder et al., 2015)
	Brush-tailed rock wallabies	PCR	0	13	None	(Stalder et al., 2015)
Serological testing						
Macropodid herpesvirus 1	Parma wallabies	VN	4	26	None	(Webber & Whalley, 1978)
	Parma and tammar wallabies	VN	1	2	None	(Webber & Whalley, 1978)
	Quokkas	VN	1	144	None	(Martínez-Pérez et al., 2021; Webber & Whalley, 1978)
	Lumholtz's tree-kangaroos	VN	4	44	None	(Shima et al., 2020)
	Woylies	VN	2	268	None	(Pacioni et al., 2013; Skogvold et al., 2017)
	Eastern grey kangaroos	VN	28	33	None	(Stalder et al., 2015; Webber & Whalley, 1978)
	Swamp wallaby	VN	1	1	None	(Stalder et al., 2015)
	Brush-tailed rock wallabies	VN	0	4	None	(Stalder et al., 2015)
	Tammar wallabies	VN	15	47	None	(Stalder et al., 2015; Webber & Whalley, 1978)
	Yellow-footed rock wallaby	VN	0	1	None	(Stalder et al., 2015)
	Rock wallabies (Petrogale species)	VN	24	106	None	(Webber & Whalley, 1978)
	Koalas	VN	0	8	None	(Stalder et al., 2015)

	Tasmanian devils	VN	1	21	None	(Stalder et al., 2015)
	Common wombats	VN	10	15	None	(Stalder et al., 2015)
	Red-necked and red-legged pademelons	VN	2	7	None	(Webber & Whalley, 1978)
	Spectacled hare-wallabies	VN	2	2	None	(Webber & Whalley, 1978)
	Hill wallaroos	VN	19	35	None	(Webber & Whalley, 1978)
	Red-necked wallabies	VN	0	6	None	(Webber & Whalley, 1978)
	Red kangaroos	VN	5	8	None	(Webber & Whalley, 1978)
	Long-nosed potoroos	VN	3	13	None	(Webber & Whalley, 1978)
	Short-nosed and long-nosed bandicoots	VN	5	17	None	(Webber & Whalley, 1978)
	Common brushtail possums	VN	0	12	None	(Webber & Whalley, 1978)
	Brown antechinus and other antechinus species	VN	1	31	None	(Webber & Whalley, 1978)
	Crest-tailed mulgara	VN	1	1	None	(Webber & Whalley, 1978)
	Fat-tailed dunnart	VN	0	1	None	(Webber & Whalley, 1978)
Macropod herpesvirus 2	Brush-tailed rock wallabies	VN	0	4	None	(Stalder et al., 2015)
	Common wombats	VN	10	15	None	(Stalder et al., 2015)
	Dorcopsis wallabies	VN	2	2	None	(Wilks et al., 1981)
	Eastern grey kangaroos	VN	28	30	None	(Stalder et al., 2015; Wilks et al., 1981)

	Goodfellow's tree kangaroos	VN	1	1	None	(Wilks et al., 1981)
	Koalas	VN	0	8	None	(Stalder et al., 2015)
	Lumholtz's tree-kangaroos	VN	4	44	None	(Shima et al., 2020)
	Parma wallabies	VN	0	2	None	(Wilks et al., 1981)
	Quokkas	VN	1	142	None	(Martínez-Pérez et al., 2021)
	Red kangaroos	VN	9	9	None	(Wilks et al., 1981)
	Red-necked wallabies	VN	0	2	None	(Wilks et al., 1981)
	Swamp wallaby	VN	1	1	None	(Stalder et al., 2015)
	Tammar wallabies	VN	1	26	None	(Stalder et al., 2015; Wilks et al., 1981)
	Tasmanian devils	VN	1	21	None	(Stalder et al., 2015)
	Tasmanian red-necked wallabies	VN	1	2	None	(Wilks et al., 1981)
	Western grey kangaroos	VN	2	2	None	(Wilks et al., 1981)
	Woylies	VN	1	68	None	(Skogvold et al., 2017)
	Yellow-footed rock wallaby	VN	0	1	None	(Stalder et al., 2015)

Hist: histopathology; EM: electron microscopy; VI: virus isolation; PCR: polymerase chain reaction; NGS: next generation sequencing; VN: virus neutralisation.

*No specific herpesvirus species was targeted as universal PCR assay was used for the testing.

Togaviridae

The togaviruses that have been detected in Australian marsupials are zoonotic and mainly transmitted by mosquitoes with marsupials serving as competent reservoirs (Old & Deane, 2005; Ong et al., 2021). The Ross River virus (RRV; a virus that causes serious public health concern), was isolated from the blood of two agile wallabies (2/17) in north Qld (Doherty et al., 1971). Antibodies to at least one of the togavirus species including RRV, Barmah Forest virus (BFV), Sindbis virus (SINV) and Getah virus (GETV) have been found in koalas, western grey kangaroos, brushtail possums, tammar wallabies, wallaroos, agile wallabies, eastern grey kangaroos, red-necked wallaby, swamp wallaby, southern brown bandicoot, long-nosed bandicoot, western native cat and quokka; but not in woylies, Parma wallabies, southern hairy-nosed wombat, brown antechinus, dusky antechinus, white-footed dunnart, and western brush wallaby (Table 2.4) (Doherty et al., 1971; Gyawali et al., 2020; Hill et al., 2009; Johansen et al., 2005; Johnson et al., 2021; Kay et al., 2007; Old & Deane, 2005; Pacioni et al., 2013; Potter et al., 2014; Vale et al., 1991). Although evidence of togaviral infections has been recorded for different Australian marsupials, clinical signs have not been seen in marsupials, thus indicating that they are potentially highly adapted reservoirs.

Papillomaviridae

Papillomaviruses are epitheliotropic and can induce benign or malignant epithelial proliferations (Mannarini et al., 2009). The full genome sequences of two novel viruses (bandicoot papillomatosis carcinomatosis virus type 1 and 2) that share genomic features of both *Papillomaviridae* and *Polyomaviridae* have been detected in the carcinomas and/or papillomas of western barred bandicoots and a southern brown bandicoot respectively (Bennett et al., 2008; Woolford et al., 2007). In another study, the complete genome of *Bettongia penicillata* papillomavirus type 1 (BpPV1) was detected in a wild adult male

woylie with multiple darkly pigmented, raised facial papillomatous lesions (Bennett et al., 2010). Papillomaviruses have been detected in healthy skin swabs from koalas (10/72) and eastern grey kangaroos (1/23) but not in red kangaroos (0/12), red-neck wallabies (0/3), swamp wallabies (0/3), whiptail wallabies (0/5), greater bilby (0/1), red-legged pademelons (0/2), ring-tail possum (0/1), brush-tail possums (0/4), and common wombat (0/1) (Antonsson & McMillan, 2006). Papillomaviruses were detected in a total of 46 marsupials out of the 250 tested (46/250). Of the 57 marsupials (57/250) showing clinical signs, 36 were confirmed positive for papillomaviruses (36/57).

Using odds ratio to analyse the aforementioned data (Appendix 1: Supplementary Table 2.4), I found a statistically significant association between papillomavirus infections and the reported papillomatous lesions ($P < 0.0001$).

Picornaviridae

The complete coding genome sequence (6.8 kb) of a novel Burpengary virus (picorna-like virus) was identified in RNA-sequencing libraries from koalas (15/26) (Harvey et al., 2019). Another study also identified the complete genome sequence (8 kb) of a novel Tasmanian devil-associated sapelovirus in the metagenome of Tasmanian devils (Chong et al., 2019). Fatal cases of encephalomyocarditis virus (*Cardiovirus A*) in two Goodfellows tree kangaroo (2/2) have been reported in Toronga zoo, Sydney (Reddacliff et al., 1997). The predominant clinical presentation and pathological lesion of the disease were sudden death and necrotizing nonsuppurative myocarditis respectively. Serological investigation of encephalomyocarditis virus (EMCV) in one red-necked wallaby, one red kangaroo, five tammar wallabies and 66 woylies showed no evidence of infection (Pacioni et al., 2013; Reddacliff et al., 1997; Rose et al., 2012).

The data presented for picornaviruses were suitable for odds ratio analysis, which showed a statistically significant association between picornavirus infections and the reported clinical signs (sudden death and necrotizing nonsuppurative myocarditis) (P = 0.0357; Appendix 1: Supplementary Table 2.4).

Flaviviridae

The family *Flaviviridae* is made up of positive sense RNA viruses that are grouped into four genera including *Hepacivirus*, *Flavivirus*, *Pegivirus*, and *Pestivirus* (Porter et al., 2020). Hepaciviruses were recently identified in a koala (koala hepaci-like virus; 1/1) suffering from severe chlamydosis, and from the archival tissues of five brushtail possums (possum hepacivirus; 5/9) using next generation sequencing technology (Chang et al., 2019; Porter et al., 2020). Antibodies to some flaviviruses including Kokobera virus (KOKV), Stratford virus (STRV), Edge Hill virus (EHV), Murray Valley encephalitis virus (MVEV), Alfuy virus (ALFV) and West Nile virus strain Kunjin (WNV_{KUNV}) have been detected in agile wallabies, eastern grey kangaroos and western grey kangaroo; but not in woylies (Table 2.4) (Doherty et al., 1971; Gyawali et al., 2020; Pacioni et al., 2013). Flaviviruses have been less studied in Australian marsupials compared to other mammals, and no clinical signs have been reported with infections in marsupials.

Poxviridae

The macropod poxviruses including eastern grey kangaroopox virus (EGKV), western grey kangaroopox virus (WGKV) and an unidentified poxviral species have been reported in eastern grey kangaroos (2/2), western grey kangaroos (2/2), quokka (1/1), and ringtail possum (1/1) respectively (Bennett et al., 2017; Papadimitriou & Ashman, 1972; Rothwell et al., 1984; Sarker et al., 2017; Vogelnest et al., 2012). The skin lesions were characterised by papules or raised nodular exophytic lesions on the face, tail, and limbs (Rothwell et al., 1984;

Sarker et al., 2017; Vogelnest et al., 2012). The detection of poxviruses has been contingent on noticeable pox lesions and only few cases have been reported in Australian marsupials.

Sedoreoviridae

Some species in the genus *Orbivirus* including *Wallal virus*, *Warrego virus*, *Eubenangee virus* and *Palyam virus* have been investigated in some marsupial species such as woylies, wallabies, kangaroos, red-necked pademelon, possums, and quokkas. The Eubenangee virus was associated with the sudden death of 120 tammar wallabies in two Australian research facilities (Rose et al., 2012). Some of these animals exhibited tachypnea, recumbency, muscle fasciculations moribundity, and death (Rose et al., 2012). This virus was isolated from the tissues of 12 affected tammar wallabies (12/14) (Rose et al., 2012). Similarly, orbiviruses of the Wallal and Warrego serogroup were implicated as the cause of blindness (viral chorioretinitis) in a large number of Australian kangaroos and wallabies (Hooper et al., 1999). Histological examination of 55 blind kangaroos showed retinitis and/or retinal degeneration, uveitis, and meningoencephalitis. Serological evidence of infection was also obtained in approximately 50% of the 437 kangaroos screened (including 23 blind kangaroos) for Wallal and Warrego viruses (Hooper et al., 1999). No serological evidence was obtained for the 344 marsupial species tested for any other sedoreoviruses (Cybinski & St George, 1982; Pacioni et al., 2013; Rose et al., 2012; Skogvold et al., 2017).

Peribunyaviridae

Serologic studies have found antibodies to Gan Gan virus (GGV) Trubanaman virus (TRUV), Koongol virus (KOOV) and Wongal virus (WONV) in eastern grey kangaroo, western grey kangaroo, quokka, red-necked wallaby, and agile wallaby; but not in brown antechinus, dusky antechinus, white-footed dunnart, southern brown bandicoot, long-nosed bandicoot, swamp wallaby, western quoll, western brush wallaby and brushtail possum (Table 2.4)

(Doherty et al., 1971; Johansen et al., 2005; Vale et al., 1991). There is evidence that some vector-borne peribunyaviruses are zoonotic and may cause polyarthritis in humans (Ong et al., 2021). There is limited knowledge on the clinical signs of these viruses in marsupials.

Arteriviridae

An arterivirus known as wobbly possum disease virus (WPDV; *Kappaarterivirus wobum*) has been associated with severe neurological disease (wobbly possum disease) in brushtail possums (Chang et al., 2019). The virus was originally identified in New Zealand, where it has been comprehensively studied (Mackintosh et al., 1995; Perrott, Meers, et al., 2000; Perrott, Wilks, et al., 2000). In Australia, the virus was detected in three possums (3/9; 33%) showing clinical signs of wobbly possum disease including nystagmus, blindness, abnormal gait, lack of pupillary light reflex, and abnormal demeanour (Chang et al., 2019). Another study provided serological evidence of WPDV in 30 Australian brushtail possums (30/188; 16%) using enzyme linked immunosorbent assay (ELISA) (Tolpinrud et al., 2020). Odds ratio showed that there was a statistically significant association between arterivirus infections and the reported clinical signs ($P = 0.00140$).

Spinareoviridae

A study examined the sera of 87 marsupials for haemagglutination-inhibition antibodies to three serotypes of mammalian orthoreovirus (Type 1 Lang, Type 2 Jones, Type 3 Dearing) and found 58 animals to be seropositive (47/72 quokkas and 11/15 kangaroos) (Stanley & Leak, 1963). The mammalian orthoreovirus has not been isolated nor associated with clinical disease in Australian marsupials; although, mild upper respiratory illness, and gastrointestinal illness have been observed in other mammals (Li et al., 2015; Narayanappa et al., 2015).

Novel viruses from the faecal virome of Tasmanian devils (*Papillomaviridae*, *Polyomaviridae*, *Herpesviridae*, *Parvoviridae*, *Circoviridae*, *Picornaviridae*, *Astroviridae*, *Sedoreoviridae* and *Picobirnaviridae*)

The Tasmanian devil is an endangered marsupial species that has been threatened with extinction by devil facial tumour disease (DFTD), a unique condition in which the tumour cells are infectious (Hamede et al., 2013). While extensive research has been conducted on DFTD, little is known about the occurrence and health implications of viruses in devils (Chong et al., 2019). Recently, novel viruses were identified in the faecal virome of Tasmanian devils using both metagenomics and metatranscriptomics sequencing approaches (Chong et al., 2019). The novel viral species identified include Tasmanian devil-associated papillomaviruses 1 and 2 (*Papillomaviridae)*, Tasmanian devil-associated polyomavirus 1 and Tasmanian devil-associated polyoma-like virus 2 (*Polyomaviridae*), dasyurid herpesvirus 3 (*Herpesviridae*), Tasmanian devil-associated chapparvoviruses 1 to 6 (*Parvoviridae*), Tasmanian devil-associated circovirus (*Circoviridae*), Tasmanian devil-associated sapelovirus (*Picornaviridae*), Tasmanian devil-associated astrovirus 1 (*Astroviridae*), Tasmanian devil-associated rotavirus 1 and 2 (*Sedoreoviridae*), and Tasmanian devil-associated picobirnaviruses 1 to 6 (*Picobirnaviridae*). Although the clinical significance of these novel viruses has not been elucidated, the baseline information provided in the study can inform future research directions and ultimately benefit conservation strategies for this endangered species.

Table 2. 4. Serological screening of vector-borne viruses in Australian marsupials.

Marsupial	**Status**	**Location**	***Togaviridae***				***Flaviviridae***						***Peribunyaviridae***				**Reference**
			RRV	BFV	SINV	GETV	KOKV	STRV	EHV	MVEV	ALFV	KUNV	GGV	TRUV	KOOV	WONV	
Agile wallaby	Wild	QLD	144/169	N/A	60/141	64/134	17/18	0/0	15/18	157/169	13/17	15/18	N/A	N/A	3/46	2/46	(Doherty et al., 1971)
Brown antechinus	Wild	NSW	0/23	0/8	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/6	0/11	N/A	N/A	(Vale et al., 1991)
Brushtail possum	Wild	NSW, QLD, WA	18/184	11/105	0/3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/3	N/A	N/A	(Hill et al., 2009; Johansen et al., 2005; Kay et al., 2007)
Dusky antechinus	Wild	NSW	0/14	0/2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/2	0/2	N/A	N/A	(Vale et al., 1991)
Eastern grey kangaroo	Wild	NSW, QLD	46/76	1/25	9/18	6/21	8/10	0/0	10/10	24/28	9/10	8/10	3/6	5/7	1/5	0/5	(Doherty et al., 1971; Vale et al., 1991)
Koala	Wild, captive	NSW, QLD	183/230	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Johnson et al., 2021; Old & Deane, 2005)
Long-nosed bandicoot	Wild	NSW	2/8	0/4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/5	0/4	N/A	N/A	(Vale et al., 1991)

Parma wallaby	Captive	NSW	0/5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Old & Deane, 2005)
Quokka	Wild	WA	0/0	2/62	0/62	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1/62	N/A	N/A	(Johansen et al., 2005)
Red-necked wallaby	Wild	NSW	6/7	0/5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1/3	1/3	N/A	N/A	(Vale et al., 1991)
Southern brown bandicoot	Wild	NSW	¼	0/5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/4	0/4	N/A	N/A	(Vale et al., 1991)
Southern hairy-nosed wombat	Captive	NSW	0/2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Old & Deane, 2005)
Swamp wallaby	Wild	NSW	5/6	0/4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/3	0/3	N/A	N/A	(Vale et al., 1991)
Tammar wallaby	Captive	NSW	34/224	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Old & Deane, 2005)
Wallaroos	Captive	NSW	4/11	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	(Old & Deane, 2005)
Western grey kangaroo	Wild	WA	1214/2732	48/228	0/228	N/A	1/100	8/100	6/100	1/100	10/100	0/100	0/0	27/128	N/A	N/A	(Gyawali et al., 2020; Johansen et al., 2005; Potter et al., 2014)
Western native cat	Wild	WA	0/0	0/6	1/6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/6	N/A	N/A	(Johansen et al., 2005)
White-footed dunnart	Wild	NSW	0/3	0/2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/2	0/2	N/A	N/A	(Vale et al., 1991)

Woylie	Wild	WA	0/73	0/72	N/A	N/A	0/73	0/73	0/73	0/73	0/73	0/73	N/A	N/A	N/A	N/A	(Pacioni et al., 2013)

RRV: Ross River virus; BFV: Barmah Forest virus; SINV: Sindbis virus; GETV: Getah virus; KOKV: Kokobera virus; STRV: Stratford virus; EHV: Edge Hill virus; MVEV: Murray Valley encephalitis virus; ALFV: Alfuy virus; KUNV: Kunjin virus; GGV: Gan Gan virus; TRUV: Trubanaman virus; KOOV: Koongol virus; WONV: Wongal virus; positive/number tested; N/A: not applicable.

2.4.4. Discussion

In this review, I observed a high occurrence rate of KoRV in Australian koalas, and this could largely be due to the endogenisation of the virus into the host genomes (Kayesh et al., 2020). Previous studies have reported 100% prevalence of KoRV provirus in Qld and lower prevalence (25-40%) in SA and Vic (Legione et al., 2017; Simmons et al., 2012). KoRV transcripts (exogenous KoRV) have been demonstrated in 100% of koalas from Qld and SA, although a small proportion (27.6%) of the SA population had missing transcripts of *pol* or *env* genes and truncated transcripts of *gag* gene (Tarlinton et al., 2022). The high KoRV seroprevalence described in this current study suggests that both exogenous and endogenous KoRVs can stimulate antibody response from hosts. Suggestive evidence implicating KoRV as the cause of diseases such as neoplasia and immunosuppression in koalas have been reported in previous studies (Hanger et al., 2000; Maher & Higgins, 2016; Tarlinton et al., 2005). By applying a statistical approach to existing published data, I found a statistically significant association between retrovirus and neoplasia, suggesting that retrovirus infection could be the primary cause of neoplasia in marsupials. However, extensive investigations including pathogenicity studies and detailed longitudinal surveys are required to establish an association of KoRV with disease and consequently aid koala management plans.

I have shown in this study that Australian marsupials are susceptible to a variety of viruses, some of which are highly pathogenic and of conservation importance. Notable among these are the herpesviruses. Herpesvirus (macropod HV1) was first isolated from the renal tissue of affected marsupials in 1975 during an outbreak of disease that led to the sudden death of a large group of captive parma wallabies (Finnie et al., 1976). Since then, similar outbreaks of fatal diseases caused by different HV species have been reported even in threatened Australian marsupial species (Table 2.3). Herpesviruses appear to be endemic in Australia as demonstrated by the widespread detection of antibodies in several asymptomatic marsupial species (Table 2.3). The virus has the capacity to form lifelong infection in susceptible hosts

where they remain latent and cause severe or fatal diseases when reactivated during stress, immunosuppression, or infection of naïve populations (Stalder et al., 2015). Given the endemicity of HVs and their potential to cause fatal diseases in Australian marsupials, there is a need to intensify disease surveillance and implement adequate preventive measures.

Marsupial viruses also pose a zoonotic or One Health concern in Australia. The RRV and BFV are important zoonotic pathogens that cause diseases in humans (Ong et al., 2021), and are the two most commonly reported arboviral infections in the Australian human population (Madzokere et al., 2022). Antibodies to RRV and BFV have been found at different prevalence rates in Australian marsupials (Table 2.4). These viruses are transmitted by mosquitoes and infection in humans can lead to clinical symptoms such as polyarthritis, rash, fever, and myalgia (Gyawali et al., 2019; Ong et al., 2021). Endemic transmission of these viruses (especially RRV) is made possible by the presence or proximity of competent marsupial reservoirs. In Qld for instance, during the dry season, wallabies move closer to human habitations, where irrigated lawns provide for an alternative food source, while more wild areas are scorched. This close proximity increases the chances of viral transmission or spill over into humans. Fortunately, the dry season also limits the breeding of the mosquito vectors. Nevertheless, this cycle of transmission could pose a great public concern since marsupials can be potential reservoirs of several important zoonotic viruses including Japanese encephalitis virus, and the local mosquito (*Culex annulirostris* Skuse) is a competent vector of JEV (Hall-Mendelin et al., 2012; Van Den Hurk et al., 2003). Disease monitoring in marsupial species using broad-based approaches will be vital for the early detection of emergence or outbreaks of potential pathogenic and zoonotic viruses (Chapter One). The KOKV, MVEV, GGV and TRUV are vector-borne zoonotic viruses that infect humans, and marsupials have been identified as potential reservoirs for these viruses (Ong et al., 2021). Therefore, it is important to consider the influence of marsupials on the spread and endemicity of vector-borne viral diseases within a One Health nexus as this would help in the control and prevention of future disease outbreaks. The vector-borne viruses in Australian

marsupials were mostly surveyed using serological methods, which may not be as sensitive and robust as the molecular based methods but would provide evidence of past infections. The use of both serological and molecular (PCR and NGS) approaches can improve the detection rate of zoonotic viruses in marsupials, and consequently provide more information on the viral epidemiological burden and inform adequate control strategies in Australia.

Surprisingly, no study reported the occurrence of an important zoonotic arbovirus, Dengue virus (DENV), in Australian marsupials, although other flaviviruses have been investigated. This is a huge gap considering the significant public and economic concerns posed by DENV. In fact, DENV has the highest incidence in humans and an exponentially increasing global distribution compared to other arboviruses (Li et al., 2022; Messina et al., 2014; Thoisy et al., 2008). DENV possesses a strict human to human urban transmission cycle and a sylvatic cycle that requires an intermediary wild mammalian (non-human) reservoir (Thoisy et al., 2008; Vasilakis et al., 2011). The urban strains are ecologically and genetically different from the sylvatic strains, and major epidemic outbreaks in humans have been caused by the urban strains (Thoisy et al., 2008; Vasilakis et al., 2011). The epidemiological and clinical significance of the sylvatic strains in terms of viral maintenance in enzootic cycles and re-emergence in human population have not been elucidated. Deployment of broad-based molecular assays such as consensus PCRs and NGS techniques in the epidemiological surveillance of DENV and other arboviruses in Australian marsupials would possibly lead to the identification of characterisation of known and novel strains and help improve our ability to understand, predict and prevent potential emergence of sylvatic viral strains in humans and wildlife.

Despite the severity of disease associated with infections, there is paucity of information regarding the epidemiology of some important marsupial viruses in Australia. For instance, the orbiviruses, WPDV and EMCV have been associated with severe or fatal diseases in Australian marsupials (Chang et al., 2019; Reddacliff et al., 1997; Rose et al., 2012).

Unfortunately, these viruses have been grossly under-studied, and there is no adequate information about the morbidity, transmission, distribution, prevalence, and control of these viruses in Australia. Similarly, several novel viruses infecting marsupials have been identified over the years, and knowledge on their pathogenic potential or epidemiological impact is still lacking. Further surveillance of marsupial populations should be conducted to determine the epidemiological implications of currently circulating marsupial viruses in Australia. Also, efforts should be geared towards the development of appropriate viral propagation cell lines to adequately predict the pathogenic and zoonotic potential of emerging novel viruses.

This study investigated the occurrence of viral infections in Australian marsupials and described the associated clinical and epidemiological significance. Noteworthy, the objectives of the various studies that reported marsupial viruses vary among researchers, and mostly included diagnostic, survey, and research studies; hence the data presented here may only be used as an indicator of the sero- and virological occurrence of marsupial viruses. Considering the enormous threats posed by viruses to wildlife species, it is rather remarkable that very few investigations have described marsupial viral diseases for the whole continent. Nonetheless, information provided in this study would help create awareness on the zoonotic and conservation significance of marsupial viruses as well as guide future research in Australia.

2.5. Chapter Summary

Since the 1970s, several species of herpesviruses have been identified and associated with significant diseases in reptiles. Earlier discoveries placed these viruses into different taxonomic groups on the basis of morphological and biological characteristics, while advancements in molecular methods have led to more recent descriptions of novel reptilian herpesviruses, as well as providing insight into the phylogenetic relationship of these viruses. Herpesvirus infections in reptiles are often characterised by non-pathognomonic signs including stomatitis, encephalitis, conjunctivitis, hepatitis, and proliferative lesions. With the exception of fibropapillomatosis in marine turtles, the absence of specific clinical signs has fostered misdiagnosis and underreporting of the actual disease burden in reptilian populations and hampered potential investigations that could lead to the effective control of these diseases. In addition, complex life histories, sampling bias and poor monitoring systems have limited the assessment of the impact of herpesvirus infections in wild populations and captive collections. In the first part of this chapter, I reviewed the current published knowledge of the taxonomy, pathogenesis, pathology, and epidemiology of reptilian herpesviruses.

Different viruses infecting marsupials have been described in Australia. These viruses include marsupial specific viruses as well as vector-borne zoonotic viruses. The second part of this chapter provides an update on the occurrence of viral infections in Australian marsupials and their associated clinical significance. A scoping review was conducted using the framework described by Arksey and O'Malley. In this review, I analysed articles that were obtained from a comprehensive literature search to provide information on the occurrence and clinical significance of viruses infecting marsupials in Australia. The viruses infecting marsupials have been detected at different rates with reported clinical diseases ranging from mild to severe. I did odds ratio where possible and found statistical association between the reported clinical disease and presence of viral pathogens (which was not previously recorded in the literature). However, epidemiological information is still lacking for some of these viruses;

future studies should focus on the pathogenic and epidemiological characterisation (including transmission, seasonality and potential impact on population) of known and novel marsupial viruses using broad-based molecular tools to help inform conservation strategies as well as identify potential risks to humans.

2.6. My Contributions to this work

- I, in collaboration with my advisors conceived and designed this review.
- I collected and reviewed data from the published literature and collated it into one table.
- I, under the advice of my supervisor, collected published DNA polymerase sequences from the family *Herpesviridae* to generate the phylogenetic tree in this chapter.
- I drafted the manuscript, analysed the data, and prepared figures and tables.
- I managed the process of journal submission and review.

Chapter Three: Development of Subfamily-based Consensus PCR Assays for the Discovery of Herpesviruses

3.1. Background and Aims

Current diagnostic methods used for the discovery of novel HVs are faced with numerous challenges including low sensitivity and high cost, which in turn could limit sample size, reduce detection rate and reliability of results. I sought to overcome these challenges and improve HV detection by developing a broad-based detection system that uses universal polymerase chain reaction (PCR) technology to detect HVs at the subfamily level (alpha-, beta- and gamma-herpesviruses). This approach is highly cost effective and will enable the sensitive detection of new HVs. This will be particularly useful for disease investigations in wildlife species where potential pathogens are largely unknown. These assays were developed in collaboration with notable Australian laboratories including the Australian Centre for Disease Preparedness (ACDP, formerly known as AAHL) and Victorian Infectious Diseases Reference Laboratory (VIDRL).

The aims of this chapter were to:

1. design sensitive consensus primers that can amplify conserved regions of the DNA polymerase gene of alpha- and gamma-HVs, and glycoprotein B gene of beta-HVs.
2. optimise the sensitivity and specificity of consensus PCR assays (STC-PCR) using a wide range of HVs across the three subfamilies.
3. further test a wide range of HVs at the ACDP laboratory with the newly developed consensus PCR assays, and validate the use of these tests in an independent laboratory.

3.2. Publication arising from this chapter

Okoh, G.R., Lockhart, M., Grimsey, J., Whitmore, D., Ariel E., Butler, J. and Horwood P.F. (2023). Development of Subfamily-Based Consensus PCR Assays for the Detection of Human and Animal Herpesviruses. *European Journal of Clinical Microbiology and Infectious Diseases*. Doi: 10.1007/s10096-023-04605-w

3.3. Introduction

Herpesviruses (HVs) are known to have a wide host range, infecting both vertebrate and invertebrate species (Kaján et al., 2020; Whitley, 1996). The virus is made up of a linear, monopartite, double-stranded DNA genome that encodes up to 300 genes and ranges from 124 to 241 kbp in length (Chaitanya, 2019). Herpesviruses are divided into three subfamilies, the *Alpha*-, *Beta*-, and *Gamma-herpesvirinae* on the basis of biological and molecular properties (Whitley, 1996). A common feature among all of the sub-groups of HVs is their ability to cause latent infection in infected hosts, which can be reactivated to cause serious illness in immuno-compromised hosts (Whitley, 1996). Clinical diseases associated with active or recrudescent HV infections vary according to the hosts and the infecting viral species. For instance, the human HVs (HHV-1 to HHV-8) are members of *Alpha*-, *Beta*- and *Gamma-herpesvirinae*, and have been associated with gingivostomatitis, herpetic keratitis, encephalitis, varicella, mononucleosis, lymphoproliferative malignancy, roseola and sarcoma (Whitley, 1996). The HVs of ruminants belong to the subfamilies *Alpha*- and *Gamma-herpesvirinae*, and infections are associated with rhinotracheitis (ovine HV1, caprine HV1), herpes mammalitis (bovine HV2), meningoencephalitis (bovine HV5), fatal systemic infection (caprine HV1), malignant catarrhal fever (ovine HV2, alcelaphine HV1, 2), ocular disease (cervine HV1), and fatal neurological disorder (bubaline HV1) (Engels & Ackermann, 1996). The avian and reptilian HVs have so far only been assigned to the subfamily *Alphaherpesvirinae* causing clinical and economic important diseases such as Marek's disease (gallid HV2) and infectious laryngotracheitis (gallid HV1) in poultry, duck plaque enteritis (anatid HV1) in waterfowl, Pacheco's disease (psittacid HV1) in psittacines, and fibropapillomatosis (chelonid HV5) in sea turtles (Boodhoo et al., 2016; Dhama et al., 2017; Gowthaman et al., 2020; Jones et al., 2016; Katoh et al., 2010; Okoh et al., 2021). Mixed infections of HV species can occur in susceptible hosts leading to a variety of clinical symptoms that may be difficult to diagnose or treat (Gruffat & Manet, 2018; Kaneko et al., 2008; Olson & Huntington, 2009; Razonable & Paya, 2002; Taj & Jamil, 2018). Therefore,

there is a need for a sensitive assay that can reliably detect HV species of more than one subfamily in the same clinical samples.

Molecular surveys often employ consensus PCR assays to detect known and novel HVs (Ehlers et al., 1999; Li et al., 2001; Porto et al., 2021; VanDevanter et al., 1996). In fact, several new HV species of mammals, reptiles and avians have been discovered using this approach (Andersson et al., 2021; Coverdill et al., 2016; Ehlers et al., 1999; Licheri & Origgi, 2020; Maboni et al., 2022; Sano et al., 2015; Smith et al., 2008). Despite these valuable outcomes, some of the existing consensus PCR assays have variable sensitivity to different HV subfamilies and require a nested PCR format, which can be costly and prone to contamination. Here, I have designed singleplex touchdown consensus PCRs (STC-PCRs) that amplify regions of the DNA polymerase (DPOL) gene of alpha- and gamma-HVs, and glycoprotein B (gB) gene of beta-HVs. This non-nested PCR assay was successfully used to detect a wide range of HVs across a broad range of herpesviruses in two independent laboratories.

3.4. Materials and Methods

3.4.1. Primer design

Degenerate consensus primers were designed for each subfamily based on the alignment of full and partial nucleotide sequences of HVs obtained from GenBank (Appendix 2: Supplementary Table 3.1). The HV sequences were imported into Geneious 11.1.5 (https://www.geneious.com) and alignments were conducted with ClustalW 2.1 using the default parameters. Primers (Table 3.1; Appendix 2: Supplementary Table 3.2) were manually generated from the conserved regions following visual inspections of the alignments (Figure 3.1). Locked nucleic acids (LNAs; Qiagen) were incorporated into some primers to increase the melting temperature and specificity. In addition, Consensus Degenerate Hybrid Oligonucleotide primers (CODEHOP) were designed with j-CODEHOP (using default parameters; Appendix 2: Supplementary Table 3.2) by importing complete and partial amino

acid sequences of HVs from GenBank into Base-by-Base primer design software (Boyce et al., 2009; Rose et al., 2003; Tu et al., 2018).

3.4.2. DNA preparation and Singleplex Touchdown Consensus PCR

Viral DNAs were extracted from infected tissues or culture supernatants using the DNeasy Blood and Tissue Kit (Qiagen) as recommended by the manufacturer. Additional DNA extracts were obtained from the Victorian Infectious Diseases Reference Laboratory (VIDRL), University of Queensland, and Berrimah Veterinary laboratory.

Following assay optimisation (data not shown), the STC-PCR was used to amplify HV DNA in a 20 µL reaction. The reaction mix contained 2 µL of DNA template, 1 µM (beta-HV) or 2 µM (alpha-HV and gamma-HV) primers (Table 3.1), 200 µM of each deoxynucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.5 U of HotStarTaq polymerase and 1x PCR buffer (Qiagen). The assays were successfully evaluated with ready-to-use pre-mixes including the GoTaq Hot Start Green Master Mix (Promega) and the HotStarTaq Plus master mix (Qiagen) to ensure the assays could be used across a range of PCR chemistries (data not shown). PCR enhancers, including 5% dimethyl sulfoxide (DMSO) and tetramethylammonium chloride (15 mM; TMAC), were also added to the reaction mix. A Touchdown PCR protocol was carried out as outlined in Table 3.2. The PCR products were analysed on a 1.5% agarose gel made up of 1x TBE buffer and 1x GelRed nucleic acid stain (Biotium).

The specificity of the herpesvirus consensus assays was evaluated by testing a large number of alphaherpesviruses (n = 22), betaherpesviruses (n = 3) and gammaherpesviruses (n = 6). The assay performance was compared to another commonly used herpesvirus nested consensus PCR (VanDevanter et al., 1996). The STC-PCR relative sensitivity was tested by assaying a series of 10-fold dilutions of the DNA extracts of representative HVs from each subfamily and comparing the limit of detection (LOD) with the VanDevanter assay (VanDevanter et al., 1996). The assay specificity was also checked by testing the consensus primer pair of one subfamily with the HV DNA templates of other subfamilies. To assess the

reproducibility of the assay, herpesviruses were tested using the assays at two independent laboratories, with 22 viruses tested at James Cook University (Townsville, Queensland) and 15 viruses tested at the Australian Centre for Disease Preparedness (Geelong, Victoria).

Table 3. 1. List of consensus primers designed to amplify the DNA polymerase (DPOL) and glycoprotein B (gB) genes of herpesviruses.

Subfamily	**Primer**	**Sequence (5'→3')**	**Orientation**	**Gene**	**Product length (bp)**
Alphaherpesvirinae	AlphaFWD1	AGCATHATYCAGGCBCAYAAYCTSTGYTTYA	Sense	DPOL	265-277
	AlphaREV2	TTRATBGCVRVCTGYTGYTTRTC	Antisense		
Betaherpesvirinae	BetaFWD_gb1	GARGCBTGGTGTHWVGATCA	Sense	gB	564
	BetaREV_gb1	YT[+C]YARR[+T]CRAANACGTT	Antisense		
Gammaherpesvirinae	GammaFWD1	GGVTAYAACRTNKSMAAYTTTGA	Sense	DPOL	650
	GammaREV1	GGRTASAGGCTRGCAAARTC	Antisense		

[+C] and [+T] indicate locked nucleic acids (LNAs).

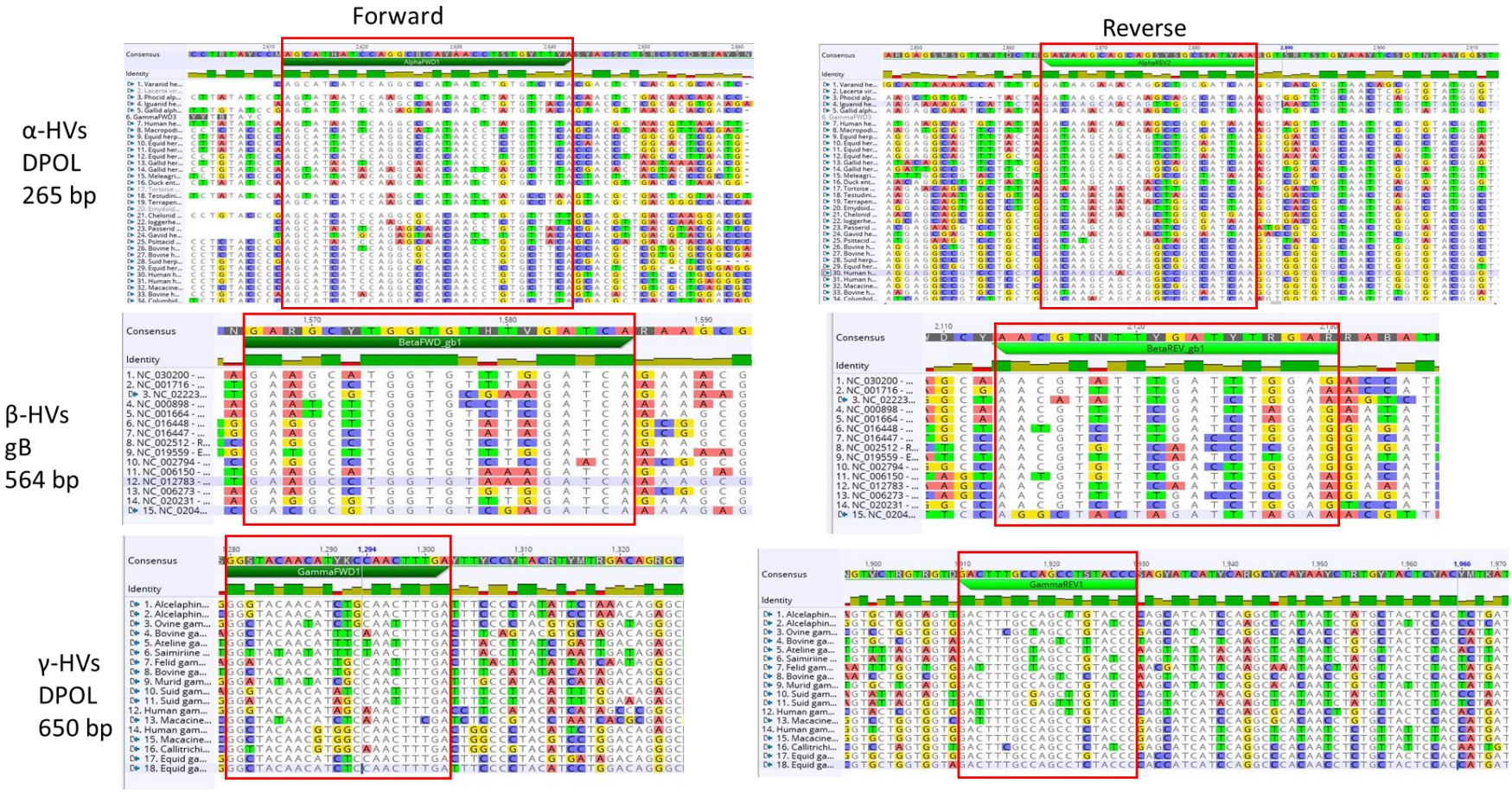


Figure 3. 1. Multiple alignments for each of the viral subfamilies showing the primer binding sites (red boxes). α-HVs= alphaherpesviruses; β-HVs= betaherpesviruses; γ-HVs= gammaherpesviruses; DPOL= DNA polymerase gene; gB= glycoprotein B gene.

Table 3. 2. Optimised touchdown PCR cycling parameters for the amplification of herpesviruses.

	Temperature (°C)	Duration	Comments
1. Initial denaturation	95	15 min	Denaturation time vary with master mixes (according to the manufacturers' instruction)
15 cycles of:			
2. Denaturation	94	1 min	
3. Annealing	63 decrements by 1°C per cycle **(-1°C/cycle)**	1 min	
4. Extension	72	2 min	
35 cycles of:			
5. Denaturation	94	1 min	
6. Annealing	48	1 min	
7. Extension	72	2 min	
1 cycle of:			
8. Final extension	72	10 min	
1 cycle of:			
9. Hold	4	Infinity	

3.5. Results

Overall, a total of 56 primers targeting the conserved regions of different HV genes were designed and tested with a wide range of HV DNAs (Table 3.3; Appendix 2: Supplementary Table 3.3). Of these, the three primer pairs recorded in Table 3.1 were found to sensitively amplify the DNA sequences of 32 HV species (Table 3.2). In addition, appropriately sized (specific) single bands were seen (for most of the HVs tested) on agarose gel following electrophoresis (Figure 3.2). The addition of 5% DMSO and 15 mM TMAC greatly improved the sensitivity, specificity, and reproducibility of the PCR reaction (Figure 3.3).

The detection limits (relative) of the STC-PCR were comparable or lower when compared to the previously reported nested-PCR (Table 3.2); except for human betaherpesvirus 6, for which the nested PCR detected the viral DNA at one 10-fold dilution lower (Figure 3.4). The STC-PCR assays produced much 'cleaner' DNA gels than the nested-PCR, which consistently produced many non-specific bands.

Subfamily assay specificity tests showed that the primer pair of one subfamily did occasionally cross-amplify HV DNAs of the other subfamilies (Figure 3.5). For instance, the alpha-HV primer pair (AlphaFWD1 and AlphaREV2) amplified the DNA of HHV-6 (faint band observed), a member of the subfamily *Betaherpesvirinae*, but did not amplify any gamma-HV DNA (Figure 3.5). The beta-HV primer pair amplified the DNA of a gamma-HV, HHV-4 (faint band), but none of the alpha-HVs. The gamma-HV primers produced varying sized bands for some alpha-HVs including crocodyline HV1 (CrHV-1), meleagridid HV1 (MeHV-1), equine HV4 (EHV-4), bovine HV1 (BoHV-1), HHV-1 and HHV-2 (Figure 3.5). None of the beta-HV DNA was amplified by the gamma-HV primers (Figure 3.5).

Table 3. 3. Human and animal herpesviruses tested by STC-PCR and the limit of detection of representative viral species.

Subfamily (Genus)	Virus	Results (limit of detection*)		Sample type
		STC-PCR	Nested-PCR [19]	
Alphaherpesvirinae				
Simplexvirus	*Human alphaherpesvirus 1* [a]	+ (10^{-3})	+ (10^{-3})	Human clinical sample
	Human alphaherpesvirus 2 [a]	+	+	Human clinical sample
	Macropodid alphaherpesvirus 1 [a]	+	+	Cell culture isolate
	Macropodid alphaherpesvirus 2 [a]	+	+	Cell culture isolate
	Bovine alphaherpesvirus 2 [b]	+	+	Cell culture isolate
Iltovirus	*Gallid alphaherpesvirus 1* [b]	+	+	Cell culture isolate
Mardivirus	*Columbid alphaherpesvirus 1* [a,b]	+ (10^{-4})	+ (10^{-4})	Animal clinical sample; Cell culture isolate
	Meleagrid alphaherpesvirus 1 [a,b]	+	+	Animal clinical sample; Cell culture isolate
	Gallid alphaherpesvirus 2 [a,b]	+	+	Animal clinical sample; Cell culture isolate
	Anatid alphaherpesvirus 1 [b]	+	+	Cell culture isolate
Varicellovirus	*Bovine alphaherpesvirus 1* [a,b]	+ (10^{-5})	+ (10^{-5})	Cell culture isolate
	Human alphaherpesvirus 3 [a]	+	+	Human clinical sample
	Equid alphaherpesvirus 1 [b]	+	+	Cell culture isolate

	Equid alphaherpesvirus 3 [b]	+	+	Cell culture isolate
	Equid alphaherpesvirus 4 [a,b]	+	+	Animal clinical sample; Cell culture isolate
	Felid alphaherpesvirus 1 [a,b]	+	+	Cell culture isolate; Vaccine (F3)
Scutavirus	*Chelonid alphaherpesvirus 5* [a]	+	+	Animal clinical sample
Unassigned/Unknown	Crocodyline herpesvirus 1 [a]	+ (10^{-5})	+ (10^{-5})	Cell culture isolate
	Crocodyline herpesvirus 2 [a]	+	+	Cell culture isolate
	Crocodyline herpesvirus 3 [a]	+	+	Cell culture isolate
	Phascolarctid herpesvirus [a]	+	+	Animal clinical sample
	Avian herpesvirus	+	+	Cell culture isolate
Betaherpesvirinae				
Cytomegalovirus	*Human betaherpesvirus 5* [a]	+ (10^{-3})	+ (10^{-2})	Human clinical sample
Roseolovirus	*Human betaherpesvirus 6* [a]	+ (10^{-2})	+ (10^{-3})	Human clinical sample
	Human betaherpesvirus 7 [a]	+	+	Human clinical sample
Gammaherpesvirinae				
Lymphocryptovirus	*Human gammaherpesvirus 4* [a]	+ (10^{-3})	+ (10^{-1})	Human clinical sample
Manticavirus	*Phascolarctid gammaherpesvirus 1* [a]	+ (10^{-3})	+ (10^{-2})	Animal clinical sample
Rhadinovirus	*Human gammaherpesvirus 8* [a]	+	+	Human clinical sample
	Bovine gammaherpesvirus 4 [b]	+	+	Cell culture isolate
Percavirus	*Equid gammaherpesvirus 2* [b]	+	+	Cell culture isolate
Macavirus	*Ovine gammaherpesvirus 2* [b]	+	+	Cell culture isolate

*The limit of the detection was recorded for the representative HVs tested.

[a] Viruses tested at James Cook University (JCU) laboratory.

[b]Viruses tested at The Australian Centre for Disease Preparedness (ACDP) laboratory.

3.6. Discussion

Despite the biological and evolutionary divergence of HVs across the three subfamilies, many evolutionarily conserved core genes still persist (Mocarski Jr, 2007; Nicholas, 2000). These genes encode proteins that play essential roles in viral entry, nucleic acid synthesis and metabolism, capsid maturation, and virion egress (Mocarski Jr, 2007). The DPOL and gB genes are among the most highly conserved genes of HVs and have previously been used as biomarkers for the detection of HVs (Ehlers et al., 1999; VanDevanter et al., 1996). In this present study, a singleplex PCR assay targeting conserved genes (DPOL or gB genes) at the subfamily level was developed and successfully used to amplify a broad spectrum of human and animal HV DNAs. Also, the assay produced bright single bands on an electrophoretic gel, which is essential for downstream amplicon sequencing and identification of novel and known HVs.

The addition of 5% DMSO and 15 mM TMAC enhanced the STC-PCR by increasing product yield and ensuring assay reproducibility. High GC content is a common feature of HV genomes (Brown, 2007), and this could pose a challenge during amplification. As previously observed (Hardjasa et al., 2010; Jensen et al., 2010), DMSO assists in reducing complex secondary structures and high melting temperature (Tm) associated with GC-rich templates, which in turn reduces duplex stability and allows efficient PCR. TMAC is often recommended when using degenerate primers and helps prevent mispriming by improving stringency of the PCR (Hung et al., 1990; Kovárová & Dráber, 2000).

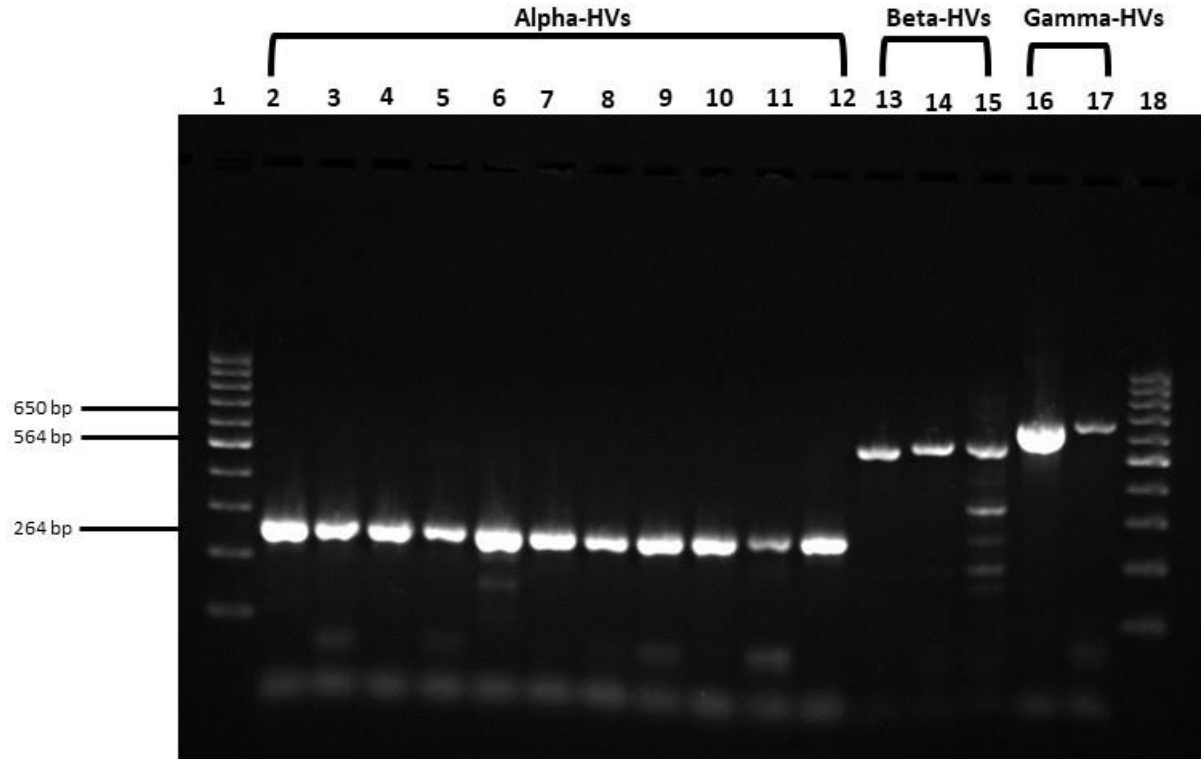


Figure 3. 2. Electrophoresis of PCR products of HV DNAs obtained by STC-PCR in a 1.5% agarose gel. Lane 1 and 18 contain a 100 bp DNA marker; Lane 2 = *Bovine alphaherpesvirus 1*; lane 3 = *Chelonid alphaherpesvirus 5*, lane 4 = *Macropodid alphaherpesvirus 1*; lane 5 = *Macropodid alphaherpesvirus 2*; lane 6 = *Human alphaherpesvirus 1*; lane 7 = *Human alphaherpesvirus 2*; lane 8 = *Human alphaherpesvirus 3*; lane 9 = *Equid alphaherpesvirus 4*; lane 10 = *Meleagrid alphaherpesvirus 1*; lane 11 = *Gallid alphaherpesvirus 2*; lane 12 = *Felid alphaherpesvirus 1*; lane 13 = *Human betaherpesvirus 5*; lane 14 = *Human betaherpesvirus 6*; lane 15 = *Human betaherpesvirus 7*; lane 16 = *Human gammaherpesvirus 4*; lane 17 = *Human gammaherpesvirus 8*.

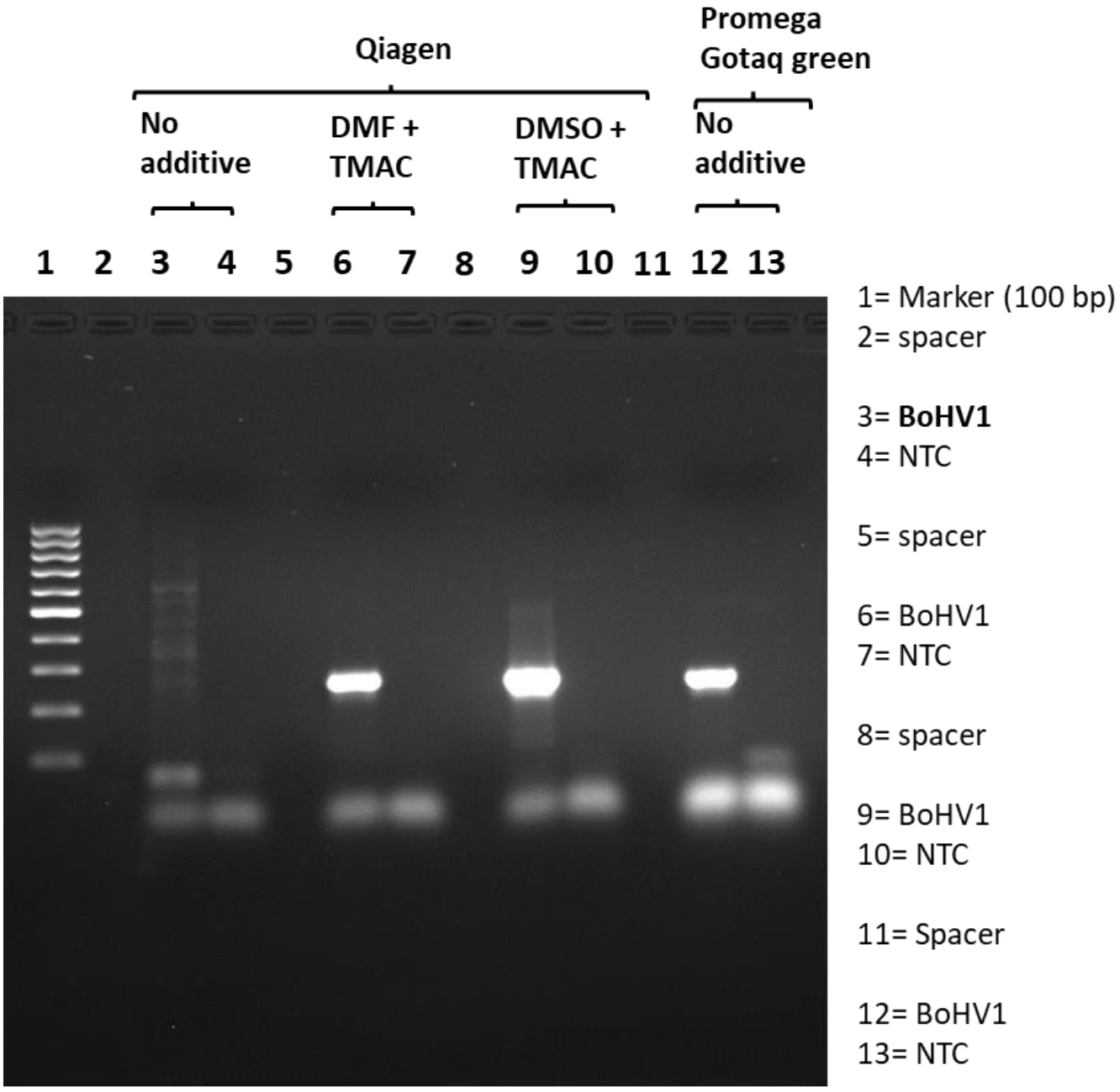
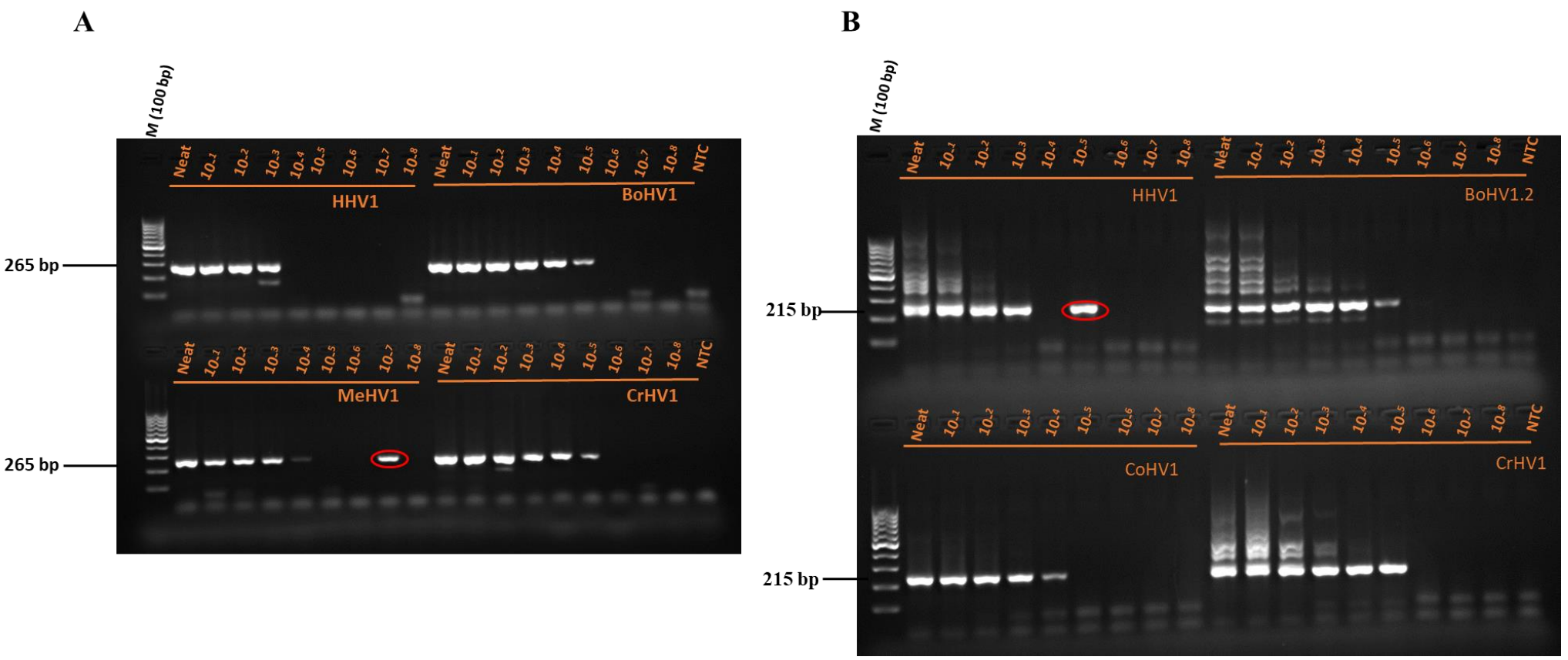
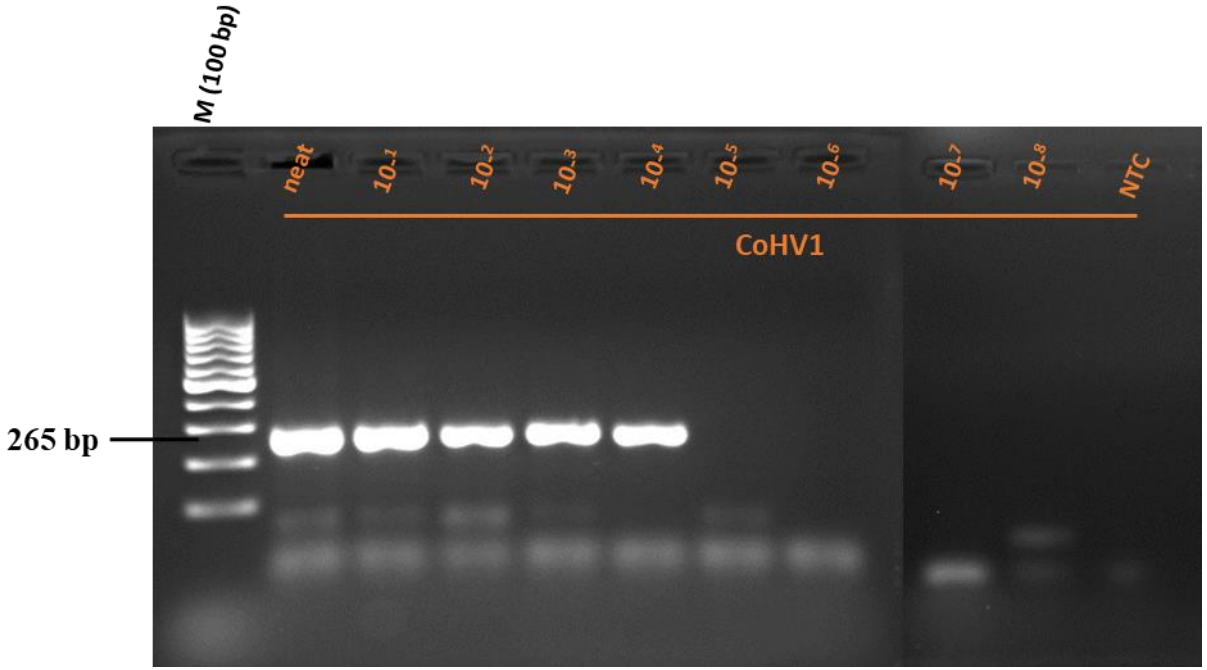


Figure 3. 3. Effect of 2.5% DMF, 5% DMSO and 15mM TMAC with AlphaFWD1 and AlphaRev2 on the detection of *Bovine alphaherpesvirus 1* (BoHV1).


A
M (100 bp)
Neat
10-1
10-2
10-3
10-4
10-5
10-6
10-7
10-8
NTC
HHV1
BoHV1
MeHV1
CrHV1
265 bp
265 bp
B
M (100 bp)
HHV1
BoHV1.2
CoHV1
CrHV1
215 bp
215 bp

C



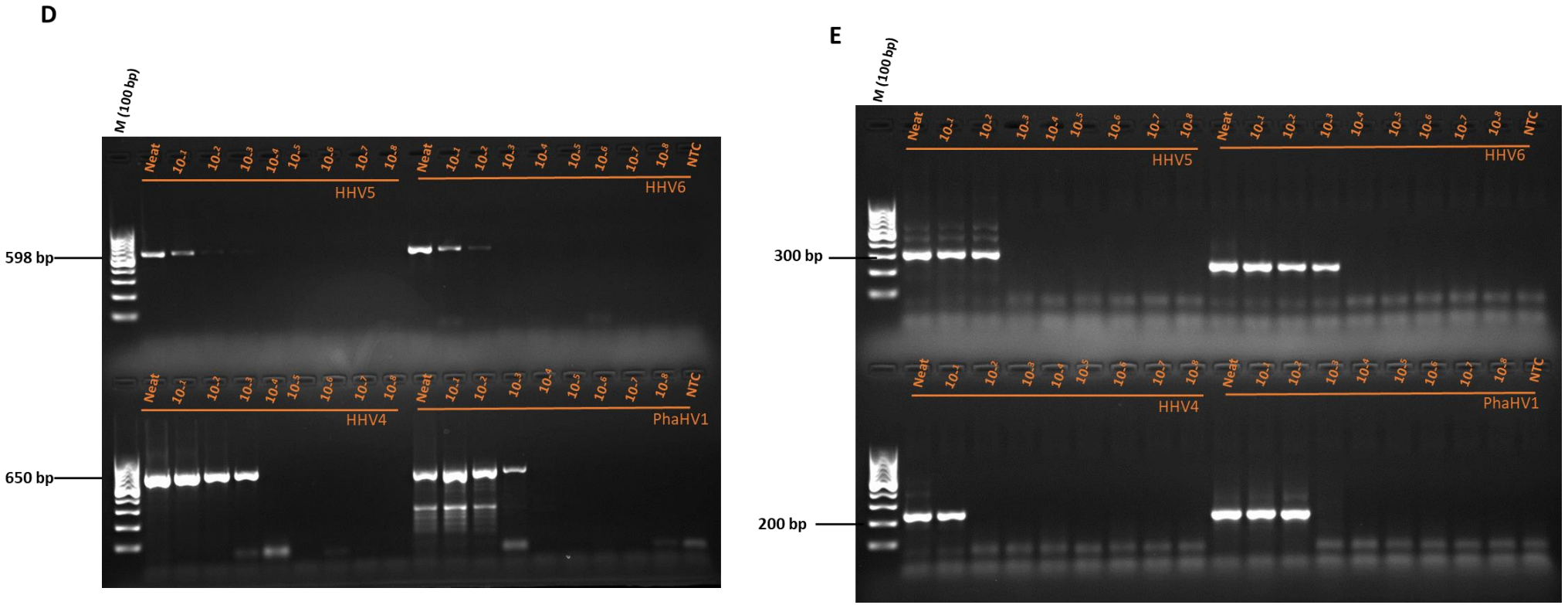
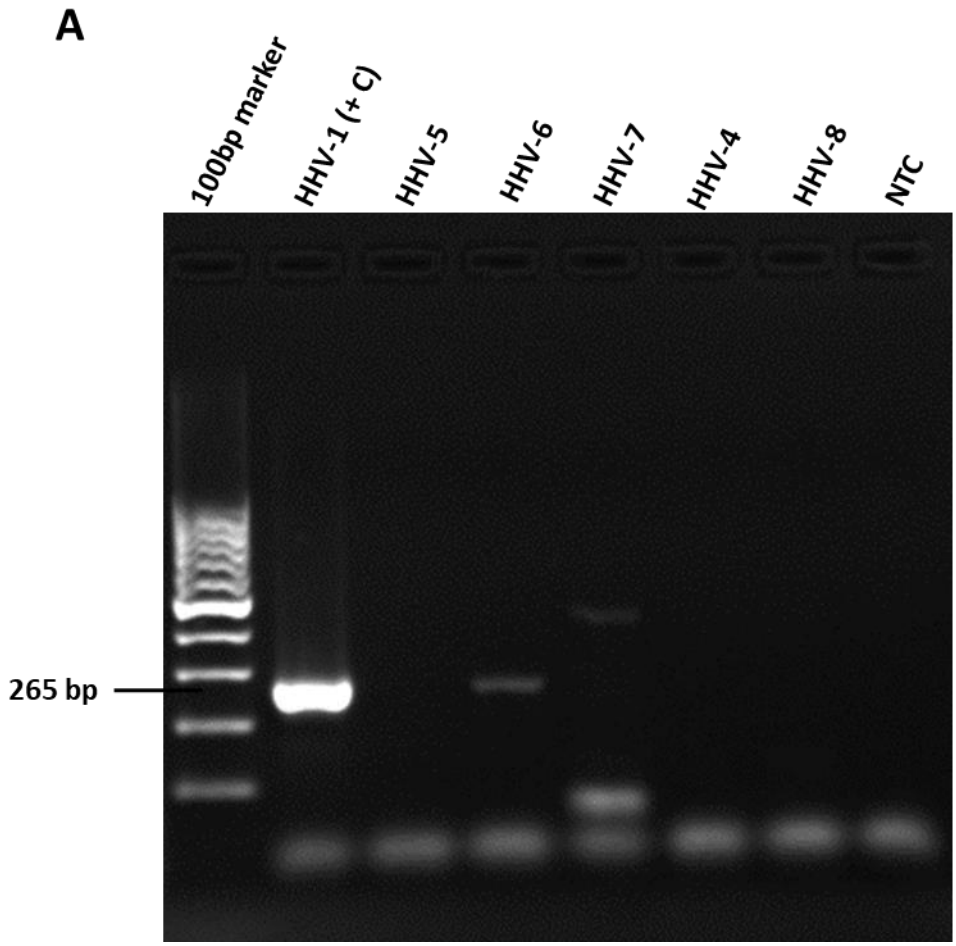
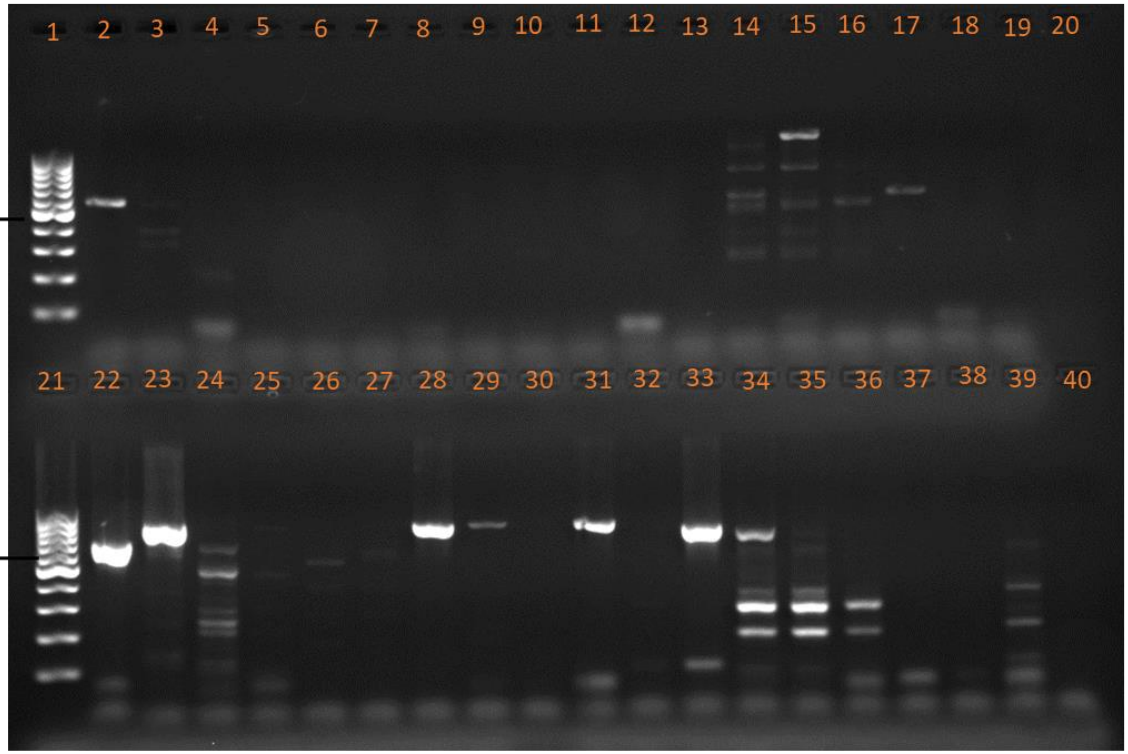


Figure 3. 4. Comparison of the detection limit (relative sensitivity) of the singleplex touchdown PCR (A, C and D) and the nested PCR (B and E) for alphaherpesviruses (HHV1= *Human alphaherpesvirus 1*; BoHV1= *Bovine alphaherpesvirus 1*; MeHV1= *Meleagrid alphaherpesvirus 1* CrHV1= Crocodyline

herpesvirus 1; CoHV1= *Columbid alphaherpesvirus*); betaherpesviruses (HH5= *Human alphaherpesvirus 5*; *Human alphaherpesvirus 6*); gammaherpesviruses (*Human alphaherpesvirus 4*; *Phascolarctid gammaherpesvirus 1*). Red cycled bands were excluded from this analysis as they were deemed to be contaminants.


A
100bp marker
HHV-1 (+ C)
HHV-5
HHV-6
HHV-7
HHV-4
HHV-8
NTC
265 bp

B



1=Marker (100 bp);
2=HHV5 (+C);
3=BoHV1; 4=ChHV5;
5=FeHV1; 6=MaHV1;
7=MaHV2; 8=HHV1;
9=HHV2; 10=HHV3;
11=EHV4; 12=MeHV1;
13=GaHV2; 14=CrHV1;
15=CrHV2; 16=CrHV3;
17=HHV4; 18=HHV8;
19=NTC; 20=spacer

BetaFWD_gb1 and BetaREV_gb1 (564 bp)

21=Marker (100 bp);
22=HHV4 (+C);
23=BoHV1; 24=ChHV5;
25=FeHV1;
26=MaHV1;
27=MaHV2; 28=HHV1;
29=HHV2; 30=HHV3;
31=EHV4; 32=GaHV2;
33=MeHV1;
34=CrHV1; 35=CrHV2;
36=CrHV3; 37=HHV5;
38=HHV6; 39=HHV7;
40=NTC

GammaFWD1 and GammaREV1 (650 bp)

Figure 3. 5. Specificity of the singleplex touchdown PCR (STC-PCR) for alphaherpesvirus (A; α-STC-PCR), betaherpesvirus (β-STC-PCR) and gammaherpesvirus (γ-STC-PCR) assays (B). The α-STC-PCR assay was used to test betaherpesviruses (HHV5=*Human alphaherpesvirus 5*; HHV6=*Human alphaherpesvirus 6;* HHV7=*Human alphaherpesvirus 7*) and gammaherpesviruses (HHV4=*Human alphaherpesvirus 4*; HHV8=*Human alphaherpesvirus 8*). The β-STC-PCR assay was used to test alphaherpesviruses (BoHV1= *Bovine alphaherpesvirus 1*; ChHV5=*Chelonid alphaherpesvirus 5*; FeHV1=*Felid alphaherpesvirus 1*; MaHV1=*Macropodid alphaherpesvirus 1*; MaHV2=*Macropodid alphaherpesvirus 2*; HHV1=*Human alphaherpesvirus 1*; HHV2=*Human alphaherpesvirus 2*; HHV3=*Human alphaherpesvirus 3;* EHV4=*Equid alphaherpesvirus 4*; MeHV1=*Meleagrid alphaherpesvirus 1*; GaHV2=*Gallid alphaherpesvirus 2*; CrHV1=Crocodyline herpesvirus 1; CrHV2=Crocodyline herpesvirus 2; CrHV3=Crocodyline herpesvirus 3) and gammaherpesviruses (HHV4 and HHV8). The γ-STC-PCR assay was used to test alphaherpesviruses (BoHV1; ChHV5; FeHV1; MaHV1; MaHV2; HHV1; HHV2; HHV3*;* EHV4; MeHV1; GaHV2; CrHV1; CrHV2; CrHV3) and betaherpesviruses (HHV5; HHV6; HHV7). Positive control (+C) and no template control (NTC) were included in all of the experiments.

In a previous study by VanDevanter et al. (VanDevanter et al., 1996), a nested PCR using degenerate primers was found to have LODs ranging from a single copy to 100 copies of HV Polymerase DNA per 100 ng of human DNA. Therefore, the sensitivity of the STC-PCR relative to the nested PCR was determined using 10-fold dilutions of representative HVs. The assays were comparable or more sensitive than the nested assay across almost all of the herpesviruses tested. With the improved sensitivity, coupled with cost and time savings, the STC-PCRs can be employed for the epidemiological and clinical detection of known and

novel HVs. Some cross-amplification between herpesvirus subfamilies was observed with the STC-PCR due to the high conservation of the targeted DPOL and gB genes at the family level. I consider this cross-amplification a universal feature of the STC-PCR for HV detection; therefore, positive results (amplicons) should be sequenced for onward identification and classification of the detected HVs.

Herpesviruses have been shown to be important pathogens across a large range of vertebrate hosts (Kaján et al., 2020). Recent initiatives to investigate viral diversity in wildlife hosts have utilised universal PCR assays to discover novel viruses, some with potential clinical and zoonotic concerns (Latimer et al., 2011; Onyuok et al., 2019). For instance, universal PCR was used to identify six novel herpesviruses in multi-infected samples of chimpanzees (*Pan troglodytes verus*) (Prepens et al., 2007). Similarly, novel herpesviruses associated with respiratory disease in birds, and hepatitis and enteritis in monitor lizards have been detected using universal PCR approaches (Hughes-Hanks et al., 2010; Shivaprasad & Phalen, 2012). Although universal PCR assays have been an invaluable tool for these viral discovery initiatives, many of these assays can be problematic due to poor sensitivity, low specificity, and contamination issues (especially with nested assays). Here I have designed and evaluated novel singleplex universal PCR assays that will be useful for detection of known and novel herpesviruses from human and animal clinical samples.

3.7. Chapter Summary

Consensus PCR assays that can be used to sensitively detect several HV species across the different subfamilies were developed in this study. Primers containing degenerate bases were designed to amplify regions of the DNA polymerase (DPOL) gene of alpha- and gamma-HVs, and the glycoprotein B (gB) gene of beta-HVs in a singleplex, non-nested touchdown PCR format. The singleplex touchdown consensus PCR (STC-PCR) was used to amplify the DNA of eight human and 24 animal HVs in two independent laboratories. The alpha, beta and gamma herpesvirus assays were consistently more sensitive than the previous published nested PCR. The findings in this study suggest that the STC-PCR assays can be employed for the discovery of novel HVs.

3.8. My Contributions to this work

- I, in collaboration with my advisors conceptualize and designed this work.
- I conducted all the bioinformatic analysis in this chapter including sequence mining, alignments, and primer design.
- I extracted DNA from animal and human clinical samples, and cell culture isolates.
- I optimised and developed the singleplex touchdown PCR protocol.
- I, in collaboration with the ACDP laboratory tested a wide variety of herpesviruses.
- I drafted the chapter and edited it as advised by collaborators and supervisors.
- I managed the process of journal submission and review.

Chapter Four: Discovery of Novel Herpesviruses and Adenoviruses in Wild Freshwater Turtles with Cutaneous Lesions

4.1. Background and Aims

Novel herpesviruses including emydoidea herpesvirus 2 (EBHV-2) and Pelusios williamsi HV have been previously identified as the cause of necrotic and proliferative cutaneous lesions in freshwater turtles (Chapter Two). In Australia, Cowan et al. (2015) and Wirth et al. (2020) reported clinical signs (cutaneous lesions) in captive and wild freshwater turtles respectively, that were consistent with infections caused by HVs, however, they were unable to identify the primary causative agents. This chapter further investigates the wild diseased population of freshwater turtles for the presence of novel HVs and other potential cutaneous lesion causing viruses (adenoviruses, papillomaviruses, and poxviruses) using the newly developed consensus PCR (Chapter Three) in combination with existing consensus PCR assays. Thus, the goals of this chapter were to:

1. identify and characterise novel HV, adenovirus, papillomavirus, and poxvirus in two wild species of Australian freshwater turtles.
2. determine an association between novel HVs and the presence of cutaneous lesions.
3. attempt to obtain the complete genome sequence of HVs directly from uncultured clinical samples.

4.2. Publication arising from this chapter

Okoh, G.R., Ariel E., Wirth, W., Whitmore D. and Horwood P.F. (2023). Molecular Detection of Novel Herpesviruses and Adenoviruses in Two Species of Australian Freshwater Turtles. *European Journal of Wildlife Research*. 69, 84 (2023). https://doi.org/10.1007/s10344-023-01714-x

4.3. Introduction

Australia is home to many freshwater turtles of the Family Chelidae and contains at least 20 endemic species (Davies & Stewart, 2013). Most Australian freshwater turtles, including the saw-shelled (*Myuchelys latisternum*) and Krefft's (*Emydura macquarii krefftii*) turtles, belong to the suborder Pleurodira, and are evolutionarily and genetically distinct from turtles in the suborder Cryptodira (Cann & Sadlier, 2017; Chiari et al., 2012). Australian freshwater turtles comprise two morphological forms, the short-necked and the long-necked turtles, which are both widely distributed across the country (Petrov et al., 2018; Scheyer, 2009; Seddon et al., 1997). They are highly aquatic species and mainly leave water to lay eggs, bask, or migrate between water bodies in search of mates or food (Brown & Brooks, 1993; Buhlmann et al., 2008). Freshwater turtles are long lived and well adapted to multiple foraging strategies, including scavenging, opportunism, herbivory and omnivory (Petrov et al., 2018). Thus, they play a vital role in maintaining ecological diversity and serve as important environmental health indicators (Lovich et al., 2018).

Many extant Australian freshwater turtles are listed as vulnerable, endangered, or critically endangered by conservation agencies (Van Dyke et al., 2019). The survival of these turtles is constantly threatened through different factors, including human activities, natural disasters, and disease outbreaks (Van Dyke et al., 2019). Viral infections can potentially threaten extinction for freshwater turtle species in local catchments, as exemplified in the Bellinger River snapping turtle (*Myuchelys georgesi*) population in 2015 (Cann et al., 2015; Spencer et al., 2018; Van Dyke et al., 2019; Zhang et al., 2018). Similarly, a novel turtle fraservirus 1 (TFV1) and the soft-shelled turtle systemic septicaemia spherical virus (STSSSV) have been reported to cause severe mortality events in freshwater turtle populations in USA and China

respectively (Chen et al., 2017; Lyu et al., 2019; Waltzek et al., 2022). Therefore, it has become pertinent to monitor the health of threatened populations for the timely detection and possible prevention of novel or emerging viral diseases.

Herpesviruses (HVs) and adenoviruses (AdVs) are important pathogens of chelonians that have been implicated in various clinical diseases with both pathogens and non-infectious factors considered as contributing in some cases (Adamovicz et al., 2018; Archer et al., 2017; Farkas & Gál, 2009; Okoh et al., 2021; Sim et al., 2016; Yonkers et al., 2015). HVs have been identified in clinically healthy chelonians due to their ability to remain latent in susceptible hosts (Aplasca et al., 2019; Lindemann et al., 2018; Marschang et al., 2015; Nieto-Claudin et al., 2022; Vincent et al., 2023; Woźniakowski & Samorek-Salamonowicz, 2015). However, HVs can cause severe diseases when reactivated in some hosts or when the virus cross-infects a closely related species (Okoh et al., 2021). HVs have been associated with necrotizing and necrotic stomatitis, glossitis, rhinitis in tortoises (Hunt, 2006; Okoh et al., 2021), proliferative and/or ulcerative lesions of the skin and shell in freshwater turtles (Okoh et al., 2021; Široký et al., 2018), and fibropapillomatosis, lung-eye-trachea disease, and grey-patch disease in sea turtles (Cárdenas et al., 2019; Jacobson et al., 1986; Robben et al., 2023; Stacy et al., 2008). AdVs that have been found to infect chelonians include members of the genera Siadenovirus, Atadenovirus and Testadenovirus (Rivera et al. 2009, Doszpoly et al. 2013; Garcia-Morante et al. 2016; Salzmann et al. 2021). While siadenovirus (Sulawesi tortoise adenovirus -1) and atadenovirus (spur-thighed tortoise adenovirus 1) have been associated with anorexia, lethargy, ulcerations and erosions of the oral mucosa, stomatitis, esophagitis, and mortality in tortoises (Rivera et al., 2009; Garcia-Morante et al. 2016), testadenoviruses have been frequently detected in clinically healthy chelonians (Doszpoly et al. 2013; Salzmann et al. 2021; Nieto-Claudin et al. 2022).

During a routine health assessment and monitoring of wild freshwater turtles at Alligator Creek, North Queensland in 2016, cutaneous lesions were observed on a large proportion of the turtles examined (Wirth et al., 2020). An investigation was conducted to identify and characterise the causative agents from lesion samples (Wirth et al., 2020). Although a specific causative agent was not identified, the pathological features of the cutaneous lesions (Figure 4.1) appear to be consistent with previously described lesions in turtles and other reptilian species associated with viral agents (detected as primary or co-pathogens) such as herpesviruses (Cowan et al., 2015; Fischer et al., 2006; Rebell et al., 1975; Shilton et al., 2016), adenovirus (Perkins et al., 2001), papillomaviruses (Jacobson et al., 1982), ranaviruses (Stöhr et al., 2013; Wirth et al., 2019), reovirus (Uĝurtas et al., 2008), and poxviruses (Buenviaje et al., 1998; Huchzermeyer et al., 1991; Jacobson et al., 1979; Penrith et al., 1991). The present study further assessed the health of freshwater turtle populations at Alligator Creek to determine the occurrence and the association of these suspected viral pathogens (herpes-, adeno-, pox- and papillomaviruses) with the cutaneous lesions. Here, we employed strategies that could improve detection, and these included the collection and testing of different sample types, modification of existing protocols as well as the design of new protocols.

Figure 4. 1. Skin lesions (red arrows) on the neck and limbs of freshwater turtles (Krefft's river and Saw-shelled turtles) at Alligator Creek.

4.4. Materials and Methods

4.4.1. Primary sampling location

Freshwater turtle sampling was conducted in Alligator Creek 25 km south of the city of Townsville, Queensland, Australia (Latitude: -19° 25' 26.00" S; Longitude: 146° 56' 44.02" E). The creek flows from Mount Elliott in Cape Bowling Green National Park to Cleveland Bay within the region of Cape Cleveland (Queensland). The lands along Alligator Creek outside the National Park have a population of approximately 1,353 people with a range of agricultural and industrial activities (Australian Bureau of Statistics, 2016). The sampling pond at the border of the national park is inhabited by two species of freshwater turtle, *Emydura macquarii krefftii* and *Myuchelys latisternum* as well as other freshwater animal species including fish, crustaceans, and crocodiles. Samples were also collected from the Ross River in Townsville, Queensland, Australia (Latitude: -19° 24' 13.19" S; Longitude: 146° 44' 1.19" E). The river is 49 km long and flows from the Hervey Range below Pepper

Pot Mountain through Townsville city and empties in the Coral Sea (http://www.bonzle.com/c/a?a=p&p=208535&cmd=sp).

To assess the association between the lesions and the potential pathogens identified in this study, we carried out a case-control investigation (Thrusfield, 2007) where the population of turtles in Alligator Creek were designated the case group. Ross River is located within 20km of Alligator Creek on a different watershed. The two waterways contain the same species of turtles and have been monitored annually for over a decade by the JCU Turtle Health Team without ever noticing lesions on the skin of turtles from Ross River similar to the ones recorded in Alligator Creek. The Ross River turtles were therefore designated the control group, and this allowed us to statistically test association between pathogens and lesions using Odds ratio (Thrusfield, 2007).

4.4.2. Sample collection and DNA extraction

Turtles were captured by hand while snorkelling and various samples were collected from 28 Krefft's river turtles (*Emydura macquarii krefftii*) and 29 saw-shelled turtles (*Myuchelys latisternum*). Additional samples were obtained from freshwater turtles (67 Krefft's and 4 saw-shelled turtles) captured by baited traps in Ross River, Townsville. For purposes of identification and disease monitoring, the captured turtles were marked by attaching a single titanium tag (National Wing Tags, Jiffy 893) to the web of one of the hind-feet.

Following the collection of morphometric data (Appendix Three: Supplementary Table 4.1), turtles were physically examined, and swabs of the oral and cloacal mucosa were collected. Lesion scrapings and swabs were collected successively from cleaned skin lesions using sterile scalpel blades and dry swabs respectively. Lastly, blood samples (up to 1 mL) were collected from the jugular vein (with 1 mL syringes and 27 G needles) into clean microfuge tubes. All of the samples were transported to the laboratory on ice and stored at -80°C until

required for testing. The blood samples were allowed to clot at room temperature in the laboratory and then centrifuged at 1000 g for 10 min. The resulting sera were then transferred into clean tubes and stored at -80°C. The oral and cloacal swabs, as well as the blood (sera and clots) samples, were initially pooled according to sample type, species, and location for an initial cost-effective screen (Tables 4.1, 4.2 and 4.3; Appendix Three: Supplementary Table 4.2). Finally, total nucleic acids were extracted from individual and pooled samples using the DNeasy Blood and Tissue Kit (Qiagen, Germany) as recommended by the manufacturer.

4.4.3. Molecular Testing for Viruses

4.4.3.1. Pooled and Lesion sample testing for viruses

The pooled and lesion samples were initially tested for herpesviruses (HVs) using a touchdown PCR with a set of consensus primers (AlphaFWD1 and AlphaREV2; Table 4.4a) targeting a 265-277 base pairs (bp) segment of the DNA polymerase gene of the subfamily *Alphaherpesvirinae* (Chapter Three). A 20 µL reaction mixture containing 2 µL of extracted DNA, 2 µM of each primer, 200 µM dNTPs, 5% DMSO, 45 mM tetramethylammonium chloride (TMAC), 1x PCR buffer and 0.5 U HotStarTaq DNA polymerase (Qiagen) was initially denatured at 95°C for 5 min and then cycled 15 times with 1 min of denaturation at 94°C, 1 min of annealing at 63°C with a decrement of 1°C per cycle (-1°C/cycle), and 2 min of extension at 72°C. This was then followed by 35 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min. After cycling, a final strand extension at 72°C for 10 min was performed and the reaction mixture was held at 4°C. Bovine HV-1 and Gallid HV-2 were used as positive controls. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen).

The pooled and lesion samples were also tested for HVs using a nested PCR, which targets a short segment (215-315 bp) of the DNA polymerase gene (VanDevanter et al., 1996) (Appendix Three: Supplementary Table 4.3). Human HV-2 was used as the positive control in this experiment. The PCR products of selected samples were cloned into pGEM®-T Easy Vector (Promega) and transformed in JM109 High Efficiency Competent Cells (Promega). The transformed plasmids were then purified with a Monarch® Plasmid Miniprep kit (NEB) as instructed by the manufacturer.

Table 4. 1. Pooled and lesion samples from Krefft's turtles (*Emydura macquarii krefftii*).

Location collected	**Sample type**	**Pool identifier**	**Number of samples per pool**	**Number of lesion samples**
Alligator Creek	Oral swab	1	28	N/A
	Cloacal swab	2	25	N/A
	Lesion swab	N/A	N/A	2
	Lesion scrapings	N/A	N/A	2
	Serum	3	9	N/A
	Serum	4	8	N/A
	Clot	5	9	N/A
	Clot	6	8	N/A
Ross River	Oral swab	7	35	N/A
	Cloacal swab	8	35	N/A

	Serum	9	12	N/A
	Clot	10	12	N/A

N/A: not applicable.

Table 4. 2. Pooled and lesion samples from saw-shelled turtles (*Myuchelys latisternum*).

Location collected	**Sample type**	**Pool identifier**	**Number of samples per pool**	**Number of lesion samples**
Alligator Creek	Oral swab	11	31	N/A
	Cloacal swab	12	32	N/A
	Lesion swab	N/A	N/A	3
	Lesion scrapings	N/A	N/A	3
	Serum	13	9	N/A
	Serum	14	8	N/A
	Clot	15	9	N/A
	Clot	16	8	N/A

N/A: not applicable.

Table 4. 3. Pools containing samples from both Krefft's and saw-shelled turtles.

Location collected	Sample type	Pool identifier	Number of each species per pool	Number of samples per pool
Ross River	Oral swab	17	Krefft's: 33 Saw-shelled: 4	37
	Cloacal swab	18	Krefft's: 31 Saw-shelled: 4	35

For the detection of AdVs, the nested PCR protocol previously described by Wellehan et al. (Wellehan et al., 2004) and modified by Anthony et al. (Anthony et al., 2013) was used in this study. Briefly, the first-round reaction mixture (20 µL total reaction volume) contained 2 µL of extracted DNA, 1 µM of each external primer (pol/F/outer and pol/R/outer), 400 µM dNTPs, 2.5 mM $MgCl_2$, 2.5 U of HotStarTaq DNA Polymerase (Qiagen), Q-solution and PCR buffer (Qiagen). Amplification of the mixture was then carried out with an initial denaturation at 95°C for 15 min, followed by 14 cycles at 94°C for 30 s, 63°C for 35 sec (-1°C/cycle), and 72°C for 60 s. Another 35 cycles at 94°C for 30 s, 45°C for 60 s and 72°C for 60 s was performed with a final strand extension at 72°C for 5 min. For the second-round amplification, 4 µL of the PCR product from the primary reaction was used with the internal primer pair (pol/F/inner and pol/R/inner) and amplified under the same conditions used for the first-round reaction.

Lesion samples were also tested for the presence of papillomavirus and poxvirus using previously described PCR protocols (Li et al., 2010; Manire et al., 2008; Mashkour et al., 2018) (Appendix Three: Supplementary Table 4.3).

PCR products and clones were sent for Sanger sequencing (Macrogen, South Korea), and only the products or clones that returned relevant sequence information were considered specific.

4.4.3.2. Consensus real-time PCR assay for herpesvirus testing of individual samples

Based on the initial HV testing of pooled samples, a new set of consensus primers (CheHV-F1 and CheHV-R1) and TaqMan probe (CheHV-P; Table 4.4) were designed (http://www.oligoarchitect.com) from the alignment (Geneious 11.1.5) of three novel sequences obtained by Sanger sequencing of the specific PCR products. The assay was designed in a region where it would be specific for the three novel HVs but would detect other closely related herpesviruses such as ChHV5 (*Scutavirus chelonidalpha5*). However, we cannot rule out that it would not detect other unknown closely related herpesviruses.

The assay was then used to amplify a 124 bp region of the three novel HVs in individual oral and cloacal swabs, and lesion samples. The optimised TaqMan probe-based PCR reaction was carried out in a 20 µL reaction mixture containing 2 µL of DNA extracts, 0.9 µM of each primer, 0.2 µM of probe, and 1x QuantiTect Probe PCR Master Mix (Qiagen). The mixture was initially denatured at 95°C for 15 minutes followed by 45 cycles of denaturation at 94°C for 15 seconds, and a combined step of annealing and extension at 60°C for 60 seconds. PCR products were sequenced at Macrogen (South Korea), and the resulting nucleotide sequences were aligned and then assigned to the original (novel) sequences based on the percentage identity score of at least 95% (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.4.4. Phylogenetic analyses

De novo assembly of the generated sequences was conducted in Geneious 11.1.5 (https://www.geneious.com) and contigs were compared with known sequences in GenBank (National Center for Biotechnology Information, Bethesda, Md.) using BLASTN. Multiple sequence alignment of the translated contigs and representative amino acid sequences available in GenBank was performed using MUSCLE (3.8.425) with the default settings. Bayesian phylogenetic analysis of the amino acid alignment was done using MrBayes plugin v3.2.6 in Geneious (Huelsenbeck & Ronquist, 2001). The analysis was performed using a fixed poisson rate matrix, gamma distributed rate variation, 4 heated chains, subsampling frequency of 200, unconstrained branch lengths, and discarding the first 25% of 1,100,000 chains as burn-in (Ossiboff et al., 2015). Phylogenetic analyses were also performed in MEGA X using the maximum likelihood (ML) method and the best model of amino acid substitution with 1000 bootstrap replications (Tamura et al. 2021; Kumar et al., 2018; Le & Gascuel, 2008).

4.4.5. Next Generation Sequencing

Total DNA of representative HV-positive samples were selected for library preparation and next generation sequencing (NGS) using Illumina NovaSeq-6000 platform at Macrogen (South Korea). Sequencing reads were trimmed to remove low quality reads and *de novo* assembled using the default parameters on CLC Genomics Workbench 22.0.2 (Qiagen). The resulting contigs were compared against the non-redundant protein databases using DIAMOND BLASTX (version 2.0.9). Contigs corresponding to HVs were extracted and subjected to BLASTN analysis to identify false positive hits and frameshifts (due to insertions or deletions). Contig extension was done using ContigExtender (Deng & Delwart, 2021). Reads were then mapped to the resulting sequences in Geneious 11.1.5, and the

sequence coding regions were identified (annotation) by BLASTX searching of nr database. Phylogenetic analyses (maximum likelihood) were performed on the identified HV sequences in MEGA X (as described above).

Table 4. 4. Primers and probe used to test for viruses in pooled or individual samples.

Oligo name	Sense	Sequence (5' to 3')	Position on Reference Sequence	Accession Number of Reference Sequence	Target Virus	Source
AlphaFWD1	Plus	AGCATHATYCAGGCBCAYAAYCTSTGYTTYA	15,732-15,762	NC_028891	Herpesvirus	(Chapter 3)
AlphaREV2	Minus	TTRATBGCVRVCTGYTGYTTRTC	15,994-15,972			
DFA	Plus	GAYTTYGCNAGYYTNTAYCC	15,711-15,730			(VanDevanter et al., 1996)
ILK	Plus	TCCTGGACAAGCAGCARNYSGCNMTNAA	15,967-15,994			

KG1	Minus	GTCTTGCTCACCAGNTCNACNCCYTT	16,453-16,428			
TGV	Plus	TGTAACTCGGTGTAYGGNTTYACNGGNGT	16,002-16,030			
IYG	Minus	CACAGAGTCCGTRTCNCCRTADAT	16,238-16,215			
CheHV-F1	Plus	TGGGCAGGGATATGCTTT	307-324	OP076950		This study
CheHV-R1	Minus	TTCATGGAGTAGGGCTGC	433-416			
CheHV-P1	Plus	[6FAM]ACATTCACGCCGTCTGGTCC[BHQ1]	340-359			
pol/F/outer	Plus	TIMGNGGIGGIMGNTGYTAYCC	5,984-5,963	NC_009989	Adenovirus	(Wellehan et al., 2004)
pol/R/outer	Minus	GTDGCRAAISHICCRTABARIGMRTT	5,429-5,454			
pol/F/inner	Plus	GTITWYGAYATHTGYGGHATGTAYGC	5,928-5,903			

pol/R/inner	Minus	CCAICCBCDRTTRTGIARIGTRA	5,608-5,630			
Low-GC_PanpoxFWD	Plus	ACACCAAAAACTCATATAACTTCT	70,398 - 70,421	NC_008291	Poxvirus	(Li et al., 2010)
Low-GC_PanpoxREV	Minus	CCTATTTTACTCCTTAGTAAATGAT	70,627-70,603			
High-GC_PanpoxFWD	Plus	CATCCCCAAGGAGACCAACGAG	120,218-120,239	NC_008030		
High-GC_PanpoxREV	Minus	TCCTCGTCGCCGTCGAAGTC	120,844-120,825			
AR-E1F2	Plus	ATGGTNCAGTGGGCNTATGA	1,672-1,691	AY904723	Papillomavirus	(Manire et al., 2008)
AR-E1R9	Minus	CATTWGTDGTDAYMAGSAKRGGVGGGCA	2,296-2,269			
Cm-Pap-109-F	Plus	GCCGATGATGTCCACTTAT	1207- 1225	EU493091.1		(Mashkour et al., 2018)
Chm-Pap-109-R	Minus	GCTGAATCCACAGAGGTAG	1315 -1297			

4.4.6. Statistical Analysis

Disease odds ratio (ψ_d) (Thrusfield, 2007) was used to determine the association between HV infections and the presence of cutaneous lesions on turtles.

4.5. Results

4.5.1. Molecular detection of viruses

Between August 2020 and May 2021, a total of 311 samples were collected from 128 freshwater turtles in Alligator Creek and Ross River, Townsville. These samples were either tested individually or in pools (Tables 4.1-4.3). As shown in Table 4.5, one pool of cloacal swabs and 16 individual oral and cloacal swabs from Krefft's turtles in Ross River tested positive for two novel HVs by PCR (Appendix Three: Supplementary Table 4.4). Six individual oral swabs from a negative pooled sample (initially tested by consensus PCR), tested positive for these novel HVs by real-time PCR (Table 4.5), indicating an increased sensitivity. None of the Krefft's turtle samples from Alligator Creek tested positive for any of the HVs. A pool of cloacal swab from Krefft's turtles obtained from Ross River was also positive for a novel AdV by consensus PCR (Table 4.5; Appendix Three: Supplementary Table 4.4). All of the individual lesion samples from Krefft's turtles tested negative for papillomavirus and poxvirus.

Of the pooled samples from saw-shelled turtles in Alligator Creek, the cloacal swab pool was positive for another novel HV (Table 4.6; Appendix Three: Supplementary Table 4.4), and upon further testing of individual swabs from this pool, 3 out of 29 were positive (Table 4.6). Three individual oral swabs (3/29) from a pool sample that initially tested negative by consensus PCR were positive for this novel HV by real-time PCR (Table 4.6). Two pools of oral and cloacal swabs from saw-shelled turtles were found to be positive for three novel AdVs (Table 4.6; Appendix Three: Supplementary Table 4.4). One lesion (1/3) swab tested

positive for HV, and none of the lesion samples was positive for AdV, papillomavirus and poxvirus (Table 4.6).

As shown in Table 4.7, one pool containing oral swabs from both Krefft's and saw-shelled turtles in Ross River tested positive for one of the novel HVs described in Table 4.5. Individual sample testing showed that seven out of the 33 oral swabs of Krefft's turtles were positive, and two out of the four saw-shelled oral swabs tested positive for the novel HVs (Table 4.7). Eleven Krefft's turtles (11/34) and all of the saw-shelled cloacal swabs (4/4) tested by real-time PCR were positive for the novel HVs, even though the initial pooled sample source was negative. None of these samples tested positive for AdV.

Overall, three pools, one lesion, 18 oral and 28 cloacal swabs from 39 of the 128 (30%) screened turtles were positive for the novel HVs. Positive results were obtained from 28 of the 95 (29%) Krefft's turtles, and 11 of the 33 (33%) saw-shelled turtles captured (Table 4.8). In Alligator Creek, seven of the 29 saw-shelled turtles were positive for HVs, while none of the Krefft's turtles tested positive. From Ross River, all of the saw-shelled turtles (4/4) and 28 out of the 67 Krefft's tested positive for HVs. Cutaneous lesions were only seen on 20 turtles (7 Krefft's and 13 saw-shelled turtles) captured from Alligator creek. Of these 20 turtles, six (30%) tested positive for HVs. Only one of the 37 turtles (21 Krefft's and 16 saw-shelled turtles) without cutaneous lesions tested positive for HV (Table 4.8). Three pooled samples from saw-shelled turtles (one each of oral and cloacal swabs) and Krefft's turtles (one cloacal swab) were positive for AdVs, and no testing was done to detect AdV in individual samples.

Table 4. 5. PCR results of samples from Krefft's turtles collected from Ross River and Alligator Creek and tested for herpesvirus, adenovirus, papillomavirus, and poxvirus.

Location Collected	**Sample Type**	**Pool identifier**	**†Herpesvirus pooled**	**††Herpesvirus individual (positive/tested)**	**†Adenovirus pooled**	**†Papillomavirus individual**	**†Poxvirus individual**
Alligator Creek	Oral swab	1	NEG	0/28	NEG	N/A	N/A
	Cloacal swab	2	NEG	0/28	NEG	N/A	N/A
	Lesion swab	N/A	N/A	0/2	NEG	0/2	0/2
	Lesion scrapings	N/A	N/A	0/2	NEG	0/2	0/2
	Serum	3	NEG	0/9	NEG	N/A	N/A
	Serum	4	NEG	0/8	NEG	N/A	N/A
	Clot	5	NEG	0/9	NEG	N/A	N/A
	Clot	6	NEG	0/8	NEG	N/A	N/A
Ross River	Oral swab	7	NEG	6/34	NEG	N/A	N/A

	Cloacal swab	8	POS	10/35	POS	N/A	N/A
	Serum	9	NEG	0/12	NEG	N/A	N/A
	Clot	10	NEG	0/12	NEG	N/A	N/A

N/A: not applicable; POS: positive; NEG: negative.

† Tested by consensus PCR assay.

†† Tested by consensus real-time PCR assay

Table 4. 6. PCR results of samples from saw-shelled turtles collected from Alligator Creek and tested for herpesvirus, adenovirus, papillomavirus, and poxvirus.

Location Collected	**Sample Type**	**Pool identifier**	**†Herpesvirus pooled**	**††Herpesvirus individual (positive/tested)**	**†Adenovirus pooled**	**†Papillomavirus individual**	**†Poxvirus individual**
Alligator Creek	Oral swab	11	NEG	3/29	POS	N/A	N/A
	Cloacal swab	12	POS	3/29	POS	N/A	N/A
	Lesion swab	N/A	N/A	1/3	NEG	0/3	0/3
	Lesion scrapings	N/A	N/A	0/3	NEG	0/3	0/3
	Serum	13	NEG	0/9	NEG	N/A	N/A
	Serum	14	NEG	0/8	NEG	N/A	N/A
	Clot	15	NEG	0/9	NEG	N/A	N/A
	Clot	16	NEG	0/8	NEG	N/A	N/A

N/A: not applicable; POS: positive; NEG: negative

† Tested by consensus PCR assay

†† Tested by consensus real-time PCR assay

Table 4. 7. PCR results of samples from Krefft's (*Emydura macquarii krefftii*) and saw-shelled (*Myuchelys latisternum*) turtles collected from Ross River and tested for herpesvirus, adenovirus, papillomavirus, and poxvirus.

Location Collected	**Sample Type**	**Pool identifier**	**†Herpesvirus pooled**	**††Herpesvirus individual (positive/tested)**	**†Adenovirus pooled**	**†Papillomavirus individual**	**†Poxvirus individual**
Ross River	Oral swab	17	POS	7/33 Krefft's	NEG	N/A	N/A
				2/4 Saw-shell			
	Cloacal swab	18	NEG	11/31 Krefft's	NEG	N/A	N/A
				4/4 Saw-shelled			

N/A: not applicable; POS: positive; NEG: negative

† Tested by consensus PCR assay

†† Tested by consensus real-time PCR assay

Table 4. 8. Detection of herpesvirus in two populations of freshwater turtles.

	Positive/Number tested				
Species	Alligator Creek		Ross River		**Total**
	Lesion*	No lesion	Lesion	No lesion	
Krefft's	0/7	0/21	0/0	28/67	28/95
Saw-shelled	6/13	1/16	0/0	4/4	11/33
Total	6/20	1/37	0/0	32/71	39/128
Disease odds ratio (ψ_d)	0.7929 (95 % CI: 0.1845 to 3.4066)		N/A	N/A	N/A

N/A: not applicable.

* A turtle was regarded as positive for 'lesions' regardless of whether they had multiple or only one lesion.

4.5.2. Statistical Analysis

The disease odds ratio (ψ_d) applied to this data indicate that there was no association between the occurrence of HVs and cutaneous lesions of freshwater turtles in Alligator Creek (0.7929; 95 % CI: 0.1845 to 3.4066).

4.5.3. Sanger sequencing and phylogenetic analysis

Initial sequencing of the PCRs product from the three pooled samples that tested positive for HVs produced relevant sequences of appropriate size (215 bp). These pools included one cloacal swab from Alligator Creek (Table 4.6) and two swabs (oral and cloacal; Tables 4.5 and 4.6) from Ross River. A modified second round nested PCR using primers DFA and IYG (VanDevanter et al. 1996) returned longer DNA polymerase fragments (413-458 bp) for two of these pools (oral and cloacal swabs from Ross River). These DNA polymerase fragments showed 81-90% similarity to each other following sequence alignments. The outputs of BLASTN analyses for the generated sequences are shown in Supplementary Table 4.4 (Appendix 3). The Bayesian phylogenetic analysis based on the amino acid sequences of

DNA polymerase revealed three novel HVs, which are placed within the subfamily *Alphaherpesvirinae* and clustered most closely to ChHV5 (YP_009207091) with a Bayesian posterior probability of 0.67 (Figure 4.2a). These viruses were tentatively named chelid herpesvirus 1 (ChelHV-1), chelid herpesvirus 2 (ChelHV-2) and chelid herpesvirus 3 (ChelHV-3) and have been accessioned in GenBank (OP076949-OP076951). Following the real-time PCR testing of individual samples and subsequent sequencing by Sanger method, relevant sequencing data were obtained for 30 of 44 submitted PCR products from 38 turtles. Of these, ChelHV-2 was detected in the oral or cloacal swabs of 22 turtles (2 saw-shelled and 20 Krefft's) while ChelHV-3 was detected in the lesion, oral or cloacal swabs samples of six saw-shelled turtles. Inadequate sequencing data returned for some samples (14 submitted PCR products) made it difficult to assign sequences to ChelHV-1.

For AdV positive pools (two saw-shelled oral and cloacal swabs from Alligator Creek and one Krefft's cloacal swab from Ross River), sequencing of the DNA polymerase PCR amplicons yielded 259 to 314 bp products (after editing sequences). A modified second round nested PCR of the positive pooled saw-shelled cloacal swab from Alligator Creek using both inner forward and outer reverse primers (Appendix Three: Supplementary Table 4.4), produced another product (459 bp) that was 100% similar to the sequence obtained for pooled Krefft's turtle cloacal swab from Ross River. Sequence alignments of all products showed three distinct polymerase sequences with 67-79% similarity to each other. The outputs of BLASTN analyses for the generated sequences are shown in Supplementary Table 4.4 (Appendix 3). Three novel AdVs that clustered with representative members of the genus *Testadenovirus* were identified following an ML phylogenetic analysis based on the amino acid sequence of DNA polymerase gene (Figure 4.3). I have tentatively named these viruses as saw-shelled turtle adenovirus 1 (SsTAdV-1), saw-shelled turtle adenovirus 2 (SsTAdV-2)

and saw-shelled turtle adenovirus 3 (SsTAdV-3). These novel sequences have been deposited in GenBank under the accession numbers OL791322, OP076952 and OP076953 respectively.

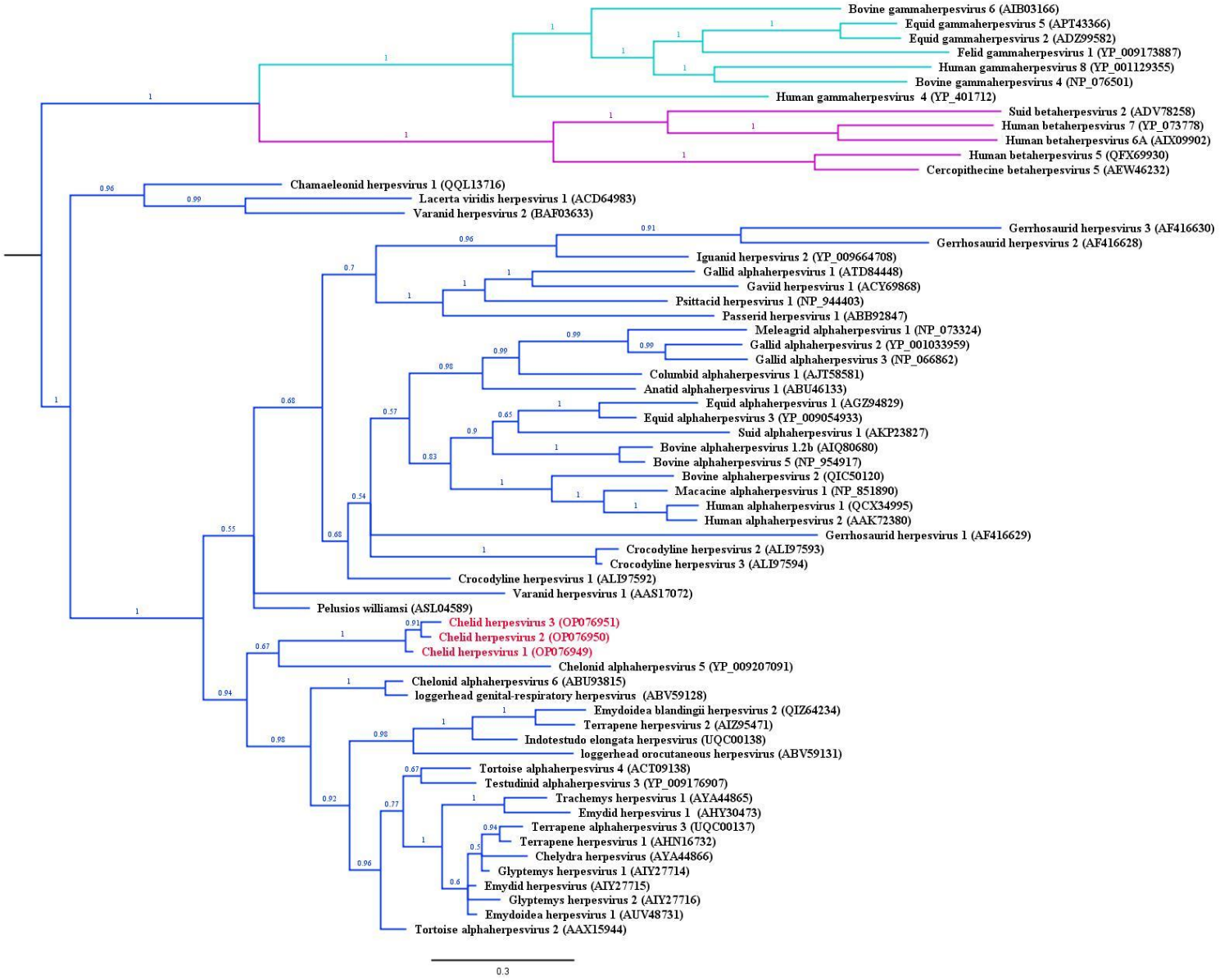

Bovine gammaherpesvirus 6 (AIB03166)
Equid gammaherpesvirus 5 (APT43366)
Equid gammaherpesvirus 2 (ADZ99582)
Felid gammaherpesvirus 1 (YP_009173887)
Human gammaherpesvirus 8 (YP_001129355)
Bovine gammaherpesvirus 4 (NP_076501)
Human gammaherpesvirus 4 (YP_401712)
Suid betaherpesvirus 2 (ADV78258)
Human betaherpesvirus 7 (YP_073778)
Human betaherpesvirus 6A (AIX09902)
Human betaherpesvirus 5 (QFX69930)
Cercopithecine betaherpesvirus 5 (AEW46232)
Chamaeleonid herpesvirus 1 (QQL13716)
Lacerta viridis herpesvirus 1 (ACD64983)
Varanid herpesvirus 2 (BAF03633)
Gerrhosaurid herpesvirus 3 (AF416630)
Gerrhosaurid herpesvirus 2 (AF416628)
Iguanid herpesvirus 2 (YP_009664708)
Gallid alphaherpesvirus 1 (ATD84448)
Gaviid herpesvirus 1 (ACY69868)
Psittacid herpesvirus 1 (NP_944403)
Passerid herpesvirus 1 (ABB92847)
Meleagrid alphaherpesvirus 1 (NP_073324)
Gallid alphaherpesvirus 2 (YP_001033959)
Gallid alphaherpesvirus 3 (NP_066862)
Columbid alphaherpesvirus 1 (AJT58581)
Anatid alphaherpesvirus 1 (ABU46133)
Equid alphaherpesvirus 1 (AGZ94829)
Equid alphaherpesvirus 3 (YP_009054933)
Suid alphaherpesvirus 1 (AKP23827)
Bovine alphaherpesvirus 1.2b (AIQ80680)
Bovine alphaherpesvirus 5 (NP_954917)
Bovine alphaherpesvirus 2 (QIC50120)
Macacine alphaherpesvirus 1 (NP_851890)
Human alphaherpesvirus 1 (QCX34995)
Human alphaherpesvirus 2 (AAK72380)
Gerrhosaurid herpesvirus 1 (AF416629)
Crocodyline herpesvirus 2 (ALI97593)
Crocodyline herpesvirus 3 (ALI97594)
Crocodyline herpesvirus 1 (ALI97592)
Varanid herpesvirus 1 (AAS17072)
Pelusios williamsi (ASL04589)
Chelid herpesvirus 3 (OP076951)
Chelid herpesvirus 2 (OP076950)
Chelid herpesvirus 1 (OP076949)
Chelonid alphaherpesvirus 5 (YP_009207091)
Chelonid alphaherpesvirus 6 (ABU93815)
loggerhead genital-respiratory herpesvirus (ABV59128)
Emydoidea blandingii herpesvirus 2 (QIZ64234)
Terrapene herpesvirus 2 (AIZ95471)
Indotestudo elongata herpesvirus (UQC00138)
loggerhead orocutaneous herpesvirus (ABV59131)
Tortoise alphaherpesvirus 4 (ACT09138)
Testudinid alphaherpesvirus 3 (YP_009176907)
Trachemys herpesvirus 1 (AYA44865)
Emydid herpesvirus 1 (AHY30473)
Terrapene alphaherpesvirus 3 (UQC00137)
Terrapene herpesvirus 1 (AHN16732)
Chelydra herpesvirus (AYA44866)
Glyptemys herpesvirus 1 (AIY27714)
Emydid herpesvirus (AIY27715)
Glyptemys herpesvirus 2 (AIY27716)
Emydoidea herpesvirus 1 (AUV48731)
Tortoise alphaherpesvirus 2 (AAX15944)
0.3

Figure 4. 2. Midpoint-rooted Bayesian phylogenetic tree of predicted amino acid sequences of herpesviral DNA-dependent DNA polymerases. Bayesian posterior probabilities are shown next to branches. The analysis involved 64 protein sequences ranging from 58-1250 amino acid residues in length. Alphaherpesviruses, betaherpesviruses, gammaherpesviruses are highlighted in blue, pink, and green branches respectively. The novel chelid herpesviruses (ChelHV1-3) are shown in red. GenBank accession numbers are shown in parentheses after the virus name. The scale bar indicates the number of amino acid substitutions per site.

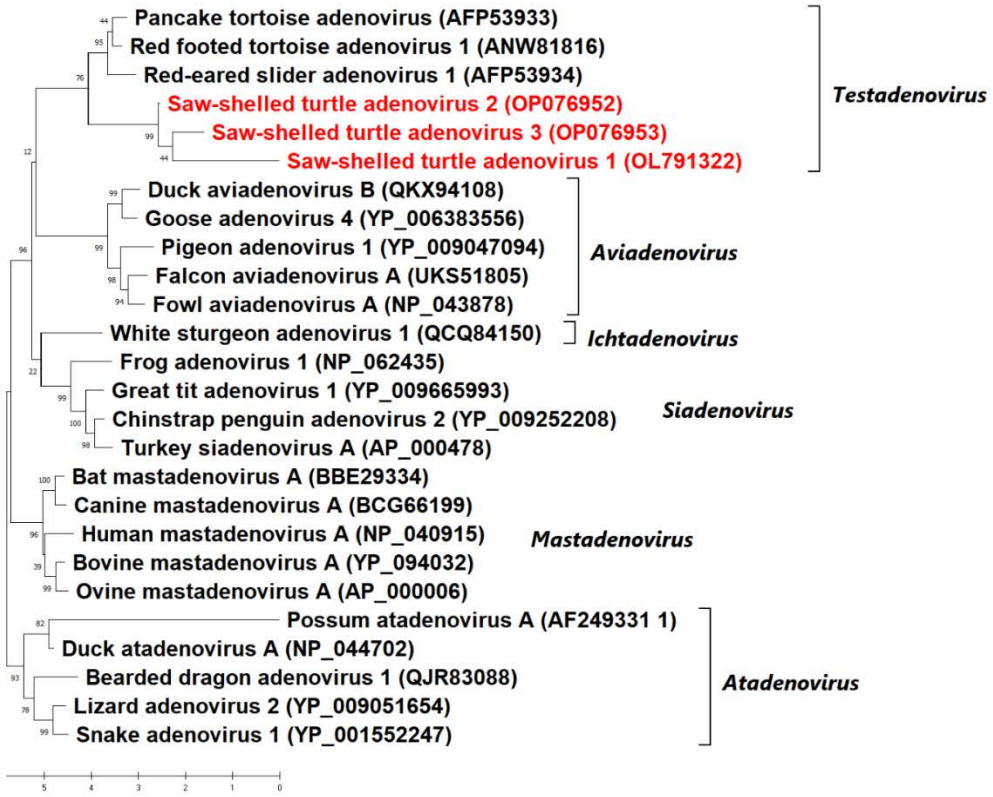


Figure 4. 3. Midpoint-rooted Maximum Likelihood tree (using LG+G model) of predicted amino acid sequences of adenoviral DNA-dependent DNA polymerases. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1000 bootstrap replications). The analysis involved 29 protein sequences (86-1341 amino acids residues in lengths). The novel adenoviruses are shown in red. GenBank accession numbers are shown in parentheses after the virus name. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11.

4.5.4. Next Generation Sequencing of chelid herpesviruses

Next generation sequencing produced 73,549,106; 71,205,296 and 68,252,498 reads for three libraries constructed from samples with C_T values of 30.47, 29.65 and 37.58 respectively. All of the sequencing data are available at the NCBI SRA under BioProject PRJNA916530. The trimmed reads were de novo assembled to produce 1,555,961, 1,566,516 and 1,850,721 contigs respectively. A total of 46 contigs (maximum length of 3300bp) were assigned to the family *Herpesviridae* following blast analysis (DIAMOND BLASTX-2.0.9). These contigs were further subjected to BLASTN analysis to remove false positive hits and identify frameshifts. Consequently, only four sequences from two libraries of ChelHV-2 and ChelHV-3 were considered adequate for read mapping and annotation. The sequences matched different genome fragments (46-54% identity; coverage depth of 1-3x) of ChHV5 (AAR28061.1, YP_010795529.1, YP_010795561.1, and YP_010795506.1), and included the partial sequences of glycoprotein B (gB; 675bp; OR030823), capsid protein (454bp; OR030824), helicase-primase primase subunit (602bp; OR030825), and uracil DNA glycosylase (UDG; 249bp; OR030826) genes (Appendix Three: Supplementary Table 4.5). There were no sequences matching the polymerase gene, hence, I could not compare these sequences with the novel sequences of ChelHVs earlier identified in this study. However, phylogenetic analyses based on the amino acid sequences of UDG and gB further confirmed the novelty and relatedness of the ChelHVs to ChHV5 (Appendix Three: Supplementary Figure 4.1).

4.6. Discussion

The identification of novel viruses (HVs and AdVs) in wild Australian freshwater turtles is critical to understanding potential threats as well as monitoring disease emergence. In this study, three novel herpesviruses were detected in healthy turtles as well as turtles with

cutaneous lesions (apart from the cutaneous lesions seen, no overt clinical signs were observed) using universal PCR assays. Several attempts to identify herpesviruses in Australian freshwater turtles by previous researchers have been unsuccessful (Cowan et al., 2015; Wirth et al., 2020). For instance, Cowan et al. (Cowan et al., 2015) obtained positive PCR amplicons for HV using a universal PCR assay and demonstrated eosinophilic intranuclear inclusion bodies in the skin lesion tissues of captive Australian Krefft's river turtles. However, they were not able to generate useful sequence data using Sanger sequencing technology. To the best of my knowledge, the finding in this study represents the first identification and partial characterisation of HV in any species of Australian freshwater turtles.

Interestingly, the novel ChelHVs form a distinct lineage with the tumour associated ChHV5 (Figure 4.2) that has been detected in the fibropapillomatous lesions of sea turtles (Mashkour et al., 2021; Page-Karjian et al., 2021; Zamana et al., 2021). The ChelHVs also clustered with other chelonian herpesviruses (Figure 4.2), some of which have been associated with cutaneous lesions. For instance, Emydoidea herpesvirus 2 and Terrapene herpesvirus 2, which share 85% sequence homology with each other, have been associated with squamous cell carcinoma (in a Blanding's turtle) and fibropapillomas (in eastern box turtles) respectively (Andersson et al., 2021; Yonkers et al., 2015). Closely related viral species are most likely to share pathogenic and epidemiologic traits (Geoghegan & Holmes, 2018; Gorbalenya & Lauber, 2017), thus implicating the ChelHVs as potential factors contributing to the pathogenesis of the cutaneous lesions seen in this study. However, PCR testing indicated that the novel ChelHVs identified in this study were not associated with the cutaneous lesions, suggesting that the ChelHVs are likely host adapted pathogens and may not be linked to the disease presentation. Previous studies have discovered HVs in clinically healthy turtles, which further indicates that HVs can cause subclinical or latent infection in

adaptive hosts (Aplasca et al., 2019; Ossiboff et al., 2015; Winter et al., 2020). Given that infection of host adapted HVs in closely related or naïve host species can cause severe disease, it is therefore necessary to further investigate the health implications of these novel ChelHVs in freshwater turtles.

All of the reptilian HVs genetically characterised to date are grouped into the subfamily *Alphaherpesvirinae* (Gatherer et al., 2021; Okoh et al., 2021). The ChelHVs also clustered within this subfamily and formed a distinct clade with ChHV5. The marked lineage variation of ChelHVs from other freshwater turtle HVs is not clearly understood. I can postulate that viruses are known to co-evolve with their hosts and most of the previously identified freshwater turtle HVs have been from Cryptodiran turtles, which are genetically and evolutionary different from the Australian species (Pleurodira), and this may have influenced the lineage variation. However, conclusions on the clustering pattern of ChelHVs cannot be drawn in this study as the phylogenetic analysis was based on the partial DNA polymerase sequences of ChelHVs. Availability of full-length sequences would allow for a more adequate or elaborate analysis, which in turn would provide a better insight into the evolutionary history and topology of reptilian HVs.

To further characterise and provide better insights into the molecular and evolutionary genomics of the novel viruses identified in this study, I attempted to sequence the complete or partial genome of ChelHV1-3 directly from clinical samples. Unfortunately, I obtained very low HV genome coverage (1-3x), recovering only four gene fragments for ChelHV-2 and 3. Sequencing non-isolated viruses is often problematic especially if the viruses are present in low amounts (Houldcroft et al., 2017; Maurier et al., 2019). These challenges can be overcome by either performing ultra-deep sequencing or primer walking the genome; however, these approaches are cost and time intensive (Houldcroft et al., 2017; Maurier et al., 2019), and beyond the scope of this study.

Herpesviruses can cause severe diseases when they infect naïve, young, or immuno-compromised reptile populations (Okoh et al., 2021; Sehrawat et al., 2018). Therefore, a good understanding of the epidemiology of HVs may aid in the prevention and control of disease outbreaks in wild and captive populations. I could not clearly differentiate the individual species of ChelHVs based on their host and location preferences, however, preliminary results suggest that the ChelHV-3 is host specific and predominantly infects the saw-shelled turtles. ChelHV-1 and 2 were mostly detected in turtles sampled from the Ross River (Appendix 3: Tables 4.4). Overall, a prevalence of 30% was recorded for ChelHVs in the two wild populations of freshwater turtles. Slightly higher prevalence rates were observed for saw-shelled (33%) than the Krefft's turtles (29%). The prevalence rates of HVs observed for saw-shelled and Krefft's turtles in this study were similar to the 31.3% reported for terrapene herpesvirus 1 in free-ranging eastern box turtles in the USA (Kane et al., 2017), but lower than the 40% and 51.5% prevalence reported in captive eastern box turtles and free-ranging bog turtles respectively (Ossiboff et al., 2015; Sim et al., 2015). Future investigations should focus on characterising the epidemiology of HVs in Australian freshwater turtles to provide better understanding of the importance of these viruses to the overall health of their hosts.

Adenoviruses have been detected in many species of reptiles and most commonly infect various species of lizard (Marschang, 2011). These viruses are distributed worldwide, and infections are characterised by anorexia, which can lead to lethargy and wasting (Ariel, 2011; Ascher et al., 2013; Marschang, 2011). Other reported clinical signs include diarrhoea, rhinorrhoea, neurological complications, stomatitis, dermatitis, and sudden death (Ariel, 2011; Bak et al., 2018; Doneley et al., 2014; Heldstab & Bestetti, 1984; Marschang, 2011; Perkins et al., 2001). In chelonians, AdVs are associated with severe systemic disease, biliverdinuria, wasting, severe bleeding, and mortality (Marschang, 2011; Rivera et al., 2009; Schumacher et al., 2012); though, some infections are asymptomatic (Franzen-Klein et al.,

2020; Nieto-Claudin et al., 2022; Vincent et al., 2023). Generally, the pathogenicity of AdVs and their ability to cause primary disease is yet to be elucidated as many clinical cases of adenoviral infections in chelonians have been associated with several cofactors such as coinfection, stress and immunosuppression (Adamovicz et al., 2018; Behncke et al., 2013; Farkas & Gál, 2009; Garcia-Morante et al., 2016; Gibbons & Steffes, 2013; Kim et al., 2002; Marschang, 2011; Rivera et al., 2009; Wilkinson, 2004). In this current study, three novel AdV species (SsTAdV1-3) were detected in two populations of freshwater turtles. Some turtles in these populations had cutaneous lesions characterised by irregular, contracted, white-tan foci on the dorsal surface of tails, legs and necks. The data obtained in this study does not support SsTAdVs as the causative agent of the cutaneous lesions. This finding is typical of members in the genus *Testadenovirus*, which have, so far, not been associated with clinical diseases (Doszpoly et al., 2013; Salzmann et al., 2021). However, the occurrence of novel AdVs in this population of freshwater turtles should not be ignored since AdVs can potentially cause diseases of conservation concerns (Jacobson et al., 1985; Marschang, 2011; Marschang et al. 2020). Therefore, there is a need to sustain disease surveillance in this population of freshwater turtles and further characterise the newly discovered viruses (SsTAdV1-3) to ascertain their pathogenicity.

Papillomavirus and poxvirus were not detected in any of the lesion samples tested in this study. Previous studies have identified these viruses as the causative agents of various cutaneous lesions in reptiles (Huchzermeyer et al., 1991; Jacobson et al., 1982; Jacobson et al., 1979; Penrith et al., 1991). Also, coinfection of these viruses with other pathogens such as HVs may cloud the causative relationship and exacerbate disease presentation (Hughes et al., 2020; Mashkour et al., 2021). My observations in this study conform to the findings of Wirth et al. (2020), thus lending support to the exclusion of papillomavirus as a possible cause of the cutaneous lesions. However, it is possible that the limited sensitivity often associated with

degenerate PCR protocols may have resulted in missed diagnosis of the virus in both studies. This assumption may also be true for the poxvirus. I further tested for papillomavirus with another assay (Mashkour et al., 2018) and still obtained negative results; though the specificity of this assay to any freshwater turtle papillomavirus (that may be associated with the lesions) was not known. Therefore, the health status of this population of freshwater turtles should be continuously assessed for the presence of potential pathogenic viruses as new and improved assays are developed.

The discovery of novel viruses in wildlife species is essential in providing insights and identifying potential factors of conservation concerns. Herpesviruses and adenoviruses are clinically important pathogens of wildlife species, though the ecology of disease in some species including reptiles remains uncertain. In this study, I identified and characterised six novel HVs and AdVs in two species of Australian freshwater turtles. Routine surveillance and further characterisation of these novel viruses may help to provide better insights into their epidemiological and conservation significance.

4.7. Data Availability Statement

The data presented in this study are openly available in GenBank and NCBI Sequence Read Archive (SRA) databases under accession numbers OP076949, OP076950, OP076951, OL791322, OP076952, OP076953, OR030823, OR030824, OR030825, and OR030826 (GenBank); and PRJNA916530 (SRA).

4.8. Chapter Summary

Cutaneous lesions were observed in a wild population of freshwater turtles during routine disease surveillance at Alligator Creek, Townsville, Australia. Previous attempts to identify the causative agent of these lesions were unsuccessful; however, existing evidence suggests viral etiology. To further investigate these events, blood samples and lesion, oral and cloacal swabs collected from 128 freshwater turtles at two locations (Alligator Creek and Ross River) were screened for herpesvirus, adenovirus, poxvirus, and papillomavirus by consensus PCR assays. I detected three novel herpesviruses (chelid herpesvirus 1-3) as well as three adenoviruses (saw-shelled turtle adenovirus 1-3). Phylogenetic analyses showed that the herpesviruses formed a distinct clade with the tumor associated chelonid alphaherpesvirus 5 within the subfamily *Alphaherpesvirinae*. The adenoviruses clustered with members of the genus *Testadenovirus*. Although the novel herpesviruses and adenoviruses could not be linked to the occurrence of cutaneous lesions, further characterization will help provide better insights into their clinical, epidemiological and conservation significance.

4.9. My contributions to this chapter

- I, in collaboration with my advisors conceptualized and designed this work.
- I collected samples from freshwater turtles with the support of my supervisors and volunteers.
- I conducted laboratory analysis including DNA extraction and PCR.
- I designed and optimised the real-time PCR protocol for HV detection.
- I conducted all the bioinformatic analysis in this chapter including sequence mining, alignments, primer design, phylogenetic analysis, and analysis of NGS data (genome assembly).

- I submitted the novel sequences to GenBank.
- I collated data tables and figures and conducted statistical analysis under the advice of my supervisors.
- I drafted the chapter and edited it as advised by collaborators and supervisors.
- I managed the process of journal submission and review.

Chapter Five: Discovery of Novel Viruses in Faecal samples of Free-Ranging Agile Wallabies using Metagenomics and Consensus PCR Approaches

5.1. Background and Aims

In Chapter Four, a viral discovery study was conducted in suspected diseased wildlife species (freshwater turtles) using consensus PCR assays. This chapter was designed to explore the use of both consensus PCR and NGS (metagenomics) approaches in a complementary manner to monitor and discover potentially pathogenic viruses in apparently healthy wildlife species. The agile wallaby (*Notamacropus agilis*) is one of the most abundant wildlife species locally (JCU and environs), and to the best my knowledge, no major disease outbreak had been reported prior or during this study (apparently healthy). Also, the adoption of a non-invasive sampling strategy ensured the suitability of this wildlife species for this study.

This aims of chapter were to:

1. identify and characterise novel viruses in the fecal samples of free-ranging agile wallabies.
2. estimate the abundance of fecal viruses of free-ranging agile wallabies.
3. assemble the genomes of novel viruses of agile wallabies.

5.2. Publications arising from this chapter

- **Okoh, G.R.,** Ariel, E., Whitmore, D. and Horwood, P.F. (in review). Metagenomic and Molecular Detection of Novel Fecal Viruses in Free-ranging agile Wallabies. *EcoHealth*.
- **Okoh, G.R**., Ariel, E., Whitmore, D. and Horwood, P.F. (2023). Draft Genome Sequence of a Novel Adenovirus Recovered from the Metagenome of Agile Wallabies. *Microbiology Resource Announcements*, e00112-23. Doi: 10.1128/mra.00112-23

5.3. Introduction

The agile wallaby (*Notamacropus agilis*) is endemic to Australia and commonly found in the northern regions of the country (Hunt et al., 2018). They often inhabit dry open woodland, heaths, dunes and grassland, and graze on grasses, shrubs, and other plants (Hunt et al., 2018; Stirrat, 2002). Pathogenic viruses have been associated with severe diseases of conservation importance in some species of wallabies (Callinan & Kefford, 1981; Finnie et al., 1976; Rose et al., 2012; Wilks et al., 1981). Despite their relative abundance in northern Australia and increased human-animal interaction, there is a paucity of epidemiological information on agile wallabies as a consequence of limited virological investigations. Characterization of the viral diversity among agile wallabies would improve our understanding of disease emergence, pathogen evolution and transmission, and potentially inform the development of vaccines or diagnostics in events of disease outbreaks.

Wild animals can harbor pathogens that may spill over to humans and domestic animals; and may also threaten the existence of endangered wild species (Ellwanger & Chies, 2021; Johnson et al., 2020; Schilling et al., 2022). For example, wallabies and other macropods are considered competent reservoirs of Ross River virus, a mosquito-borne zoonotic virus associated with serious public health and economic concerns in Australia (Skinner et al., 2021; Stephenson et al., 2018; Yuen & Bielefeldt-Ohmann, 2021). Thus, studying and monitoring diseases in wildlife populations is an important discipline within the One Health approach to disease preparedness and control (Kelly et al., 2020; Maas et al., 2016; Rugarabamu, 2021). As the interest in wildlife disease research is growing, animal welfare has been prioritized to limit distress and pain on sampled populations (Fenwick et al., 2009; Lindsjö et al., 2016; Schilling et al., 2022; Zemanova, 2020). Traditional methods of sample collection from wild or free-ranging animals involve capturing and handling in the most humane way possible. However, this approach could be time intensive, costly, and requires

special equipment, training and ethics approval, making it difficult to conduct routine sampling and disease monitoring for wild animals (Schilling et al., 2022). Non-invasive sampling has been proposed to be a viable alternative method of sample collection for disease monitoring in the wild (Baus et al., 2019; Schilling et al., 2022). This approach has been employed to detect several viruses of zoonotic and conservation importance in wild animals (Bataille et al., 2019; de Carvalho Ferreira et al., 2014; Giles et al., 2021; Hardmeier et al., 2021; Mouchantat et al., 2014; Wood et al., 2021). For these reasons, this study explored the possibility of non-invasively sampling free-ranging agile wallabies for fecal viral identification and characterization.

Advances in next generation sequencing techniques have greatly facilitated studies on viral microbiome (virome) and discovery of new viruses in humans and animals (Bexfield & Kellam, 2011; Cao et al., 2022). Metagenomics is becoming a mainstay in the field of epidemiology for disease surveillance as exemplified in the recent coronavirus disease (COVID-19) pandemic (Castañeda-Mogollón et al., 2021; Iša et al., 2022; Rodriguez et al., 2021). In medicine, it has been used to resolve difficult-to-diagnose cases wherein the causative pathogens were unknown or initial differential diagnosis remained broad (Chen et al., 2022; Hirakata et al., 2021; Kufner et al., 2019). However, missed diagnosis of low-abundant viruses coupled with false positive results due to imperfect microbial databases are some of the reported challenges associated with the metagenomics analysis (Chen et al., 2022; Rose et al., 2016). PCR based assays have been used to detect known (conventional and real-time PCR) and novel (consensus PCR) viruses in clinical and field samples (Fieldhouse et al., 2020; Luciani et al., 2021; Martínez-Pérez et al., 2021). Although PCR techniques can only detect a limited number of targets within a given sample with less discovery power, it can be used in combination with the highly sensitive Sanger sequencing to complement or verify the results of metagenomics analyses in relation to pathogen

detection (Li et al., 2021). This study aimed to identify and characterize the fecal viruses of free-ranging agile wallabies using metagenomics and PCR techniques.

5.4. Materials and Methods

5.4.1. Non-invasive sample collection

In 2021, I collected 16 fresh fecal samples belonging to free-ranging agile wallabies early in the morning from the ground at grazing sites around James Cook University and Townsville University Hospital, Townsville, Australia. The samples were collected (over a period of two days) using sterile techniques and individually placed in a plastic container, and transported on ice to the laboratory where they were stored at -80°C.

5.4.2. Viral Enrichment, Nucleic Acid Extraction and cDNA synthesis

Viral enrichment was performed according to a previously described protocol (Ramírez-Martínez et al., 2018). Briefly, individual fecal samples (1 g) were suspended in 10 ml of phosphate buffered saline (PBS) and vigorously homogenized. The homogenates (5 mL each) were grouped into three pools, with two pools (pools 1 and 3) containing five samples and a third pool (pool 2) containing six samples. The pools were centrifuged (Beckman Coulter) at 7000 ×g and 4°C for 15 min to clarify the contents. The resulting supernatants were filtered using a 0.25 µm syringe filter and ultracentrifuged (Beckman Optima MAX-XP, MLA-150 fixed-angle rotor) at 100,000 ×g and 4°C for 3 hours. The pellets were resuspended in 500 µL of Tris-HCL buffer (10 mM; pH 7.5) and treated with 20 U/mL DNase I (NEB; M0303S) and 0.2 mg/mL RNase A (NEB; T3018L) at 37°C for 60 min. Viral DNA and RNA were then simultaneously extracted using QIAamp MinElute Virus kit (Qiagen; 57704) as recommended by the manufacturer. Also, DNA was extracted from the untreated individual

fecal samples using QIAamp Fast DNA Stool Mini Kit (Qiagen; 51604) following the manufacturer's instructions.

The synthesis of viral cDNA was carried out using Revertaid first strand cDNA Synthesis kit (Thermo Fisher Scientific; K1621) following the manufacturer's instructions. Second strand cDNA synthesis was performed according to a previously published protocol (Goya et al., 2018). In brief, the second strand was synthesized with 100 U/mL DNA polymerase I, Large (Klenow) Fragment (NEB; M0210S), 400 µM dNTPs, 1.6 µM random hexamers (Thermo Fisher Scientific) and 1x NEBuffer™ 2 buffer. The mixture was then incubated at 37°C for 60 min and the reaction terminated by heating for 20 min at 75°C. The synthesized double stranded DNA was mixed with viral DNA (Zhao et al., 2018), quantified and purified using Qubit dsDNA HS assay (Invitrogen, Carlsbad, CA, USA) and Agencourt AMPure XP beads (Beckman Coulter; bead/sample ratio of 9:5) respectively.

5.4.3. Library preparation, Illumina sequencing and Bioinformatics

Library preparation and sequencing were carried out at Macrogen (Seoul, South Korea). Sequencing libraries were prepared using a Nextera DNA XT kit (Illumina) according to the manufacturer's recommendation. Libraries were then sequenced on a Novaseq6000 platform to obtain 151-bp paired end reads.

The quality of the sequencing reads was inspected with FastQC-0.11.9 (Andrews, 2010), and low quality reads were trimmed using Trimmomatic-0.39 (Bolger et al., 2014). The resulting reads were *de novo* assembled in SPAdes-3.12.0 (Bankevich et al., 2012) to produce contigs. Read pairs were also merged with fastq-join-1.3.1 (Aronesty, 2013), and reads that failed to merge were concatenated with the merged reads to avoid losing any information. The contigs and merged reads were compared against the non-redundant nucleotide and protein databases

using BLASTN (version 2.12.0) (Altschul et al., 1990; Sayers et al., 2022) and DIAMOND BLASTX (version 2.0.9) (Buchfink et al., 2021) respectively, with E-value set at 1E-3. To avoid false positive results, only hits obtained from both databases were reported in this study. Finally, taxonomic assignment and visualization of the BLAST outputs were done using krona-2.8 (Ondov et al., 2011).

For the assembly of a novel adenovirus genome, the trimmed reads from pool 3 were normalized with BBnorm v39.01 (sourceforge.net/projects/bbmap/) and de novo assembled using SPAdes v3.15.5 on careful mode. The resulting contigs were searched in Diamond BLASTX against the NCBI nr protein database to identify contigs corresponding to adenoviruses. To complete the genome assembly, reads were then mapped to the adenoviral contig in Geneious 11.1.5 (https://www.geneious.com). Prediction of open reading frames (ORFs) was performed using Glimmer3 in Geneious 11.1.5, and ORF annotations were determined by BLASTX searching against the NCBI nr protein databases.

Also, the genome of a novel picobirnavirus was assembled by mapping reads to two of the longest contigs (corresponding to each segment of the bi-segmented genome) obtained from the initial SPAdes analysis (not careful mode). The mapping was done using bowtie2 v2.3.5.1 (Langmead & Salzberg, 2012). ORFs were then predicted and annotated using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and BLASTX respectively. All bioinformatic tools were run with default parameters unless otherwise specified.

5.4.4. PCR testing and Sanger sequencing

Specific or universal PCR assays were used to screen pooled and individual fecal samples for herpesviruses (HVs) and retroviruses, and to verify some agile wallaby related viruses identified by metagenomics. Details of primers and protocols used in this study are shown in

Table 5.1. Positive PCR products were then confirmed by sequencing at Macrogen (Seoul, South Korea) by Sanger method.

5.4.5. Phylogenetic Analyses

Nucleotide sequences of the newly identified viruses were translated and aligned to representative reference viral sequences in GenBank (National Center for Biotechnology Information, Bethesda, Md.) using MUSCLE (3.8.425) (Edgar, 2004). Phylogenetic trees of the aligned amino acids (aa) were inferred in MEGA X (Kumar et al., 2018) using the maximum likelihood (ML) method and the best model of amino acid substitution with 1000 bootstrap replications.

Table 5. 1. PCR primers and protocols for testing viruses in pooled and individual samples.

Virus	**Target gene**	**Oligo**	**Primer Sequence (5' to 3')**	**Orientation**	**PCR protocol**	**Reference**
Adenovirus	DNA polymerase	pol/F/ outer pol/R /outer pol/F/ inner pol/R /inner	TIMGNGGIGGIMGNTGY TAYCC GTDGCRAAISHICCRTAB ARIGMRTT GTITWYGAYATHTGYGG HATGTAYGC CCAICCBCDRTTRTGIAR IGTRA	Sense Antisense Sense Antisense	Nested 95°C for 5 min 14 cycles [94°C for 30s, 63 °C for 35s (-1°C/cycle), 72°C for 60s] 35 cycles (94°C for 30s, 45 °C for 60s, 72°C for 60s) 72°C for 5 min	(Wellehan et al., 2004)

					Expected size: 318 to 324 bp	
Parvovirus	Non-structural protein	PV-190F PR4	GGACAACACTATAGACCCTTAAACT AGAAACCATTGAGTCGGAGG	Sense Antisense	95°C for 5 min 40 cycles (94°C for 60s, 58 °C for 60s, 72°C for 60s) 72°C for 10 min Expected size: 101 bp	This study
Polyomavirus	Major-capsid protein	VP1/lf VP1/lr VP1/2f VP1/2r	CCAGACCCAACTARRAATGARAA AACAAGAGACACAAATNTTTCCNCC ATGAAAATGGGGTTGGCCCNCTNTGYAARG CCCTCATAAACCCGAACYTCYTCHACYTG	Sense Antisense Sense Antisense	Nested 95°C for 5 min 45 cycles (94°C for 30 s, 46 °C for 60s, 72°C for 60s) 72°C for 5 min Expected size: 249-273 bp	(Johne et al., 2005)
Circovirus	Replication initiator protein	CV-F1 CV-R1 CV-F2	GGIAYICCICAYYTICARGG AWCCAICCRTARAARTCRTC GGIAYICCICAYYTICARGGITT	Sense Antisense Sense Antisense	Nested 95°C for 5 min 40 cycles (95°C for 1 min, 52 °C for 60s, 72°C for 60s) 72°C for 10 min	(Li et al., 2010)

		CV-R2	TGYTGYTCRTAICCRTCCCACCA		Expected size: 400 bp	
Herpesvirus	DNA polymerase	DFA KG1 ILK TGV IYG	GAYTTYGCNAGYYTNTAYCC GTCTTGCTCACCAGNTCNACNCCYTT TCCTGGACAAGCAGCARNYSGCNMTNAA TGTAACTCGGTGTAYGGNTTYACNGGNGT CACAGAGTCCGTRTCNCCRTADAT	Sense Sense Antisense Sense Antisense	Nested PCR 5 min at 94°C 45 cycles (94°C for 30s, 46°C for 60s 72°C for 60s) 72°C for 10 min Expected size: 215-315 bp	(VanDevanter et al., 1996)
	DNA polymerase	Alpha FWD 1 Alpha REV 2 BetaFWD_gb1 Beta REV_gb1	AGCATHATYCAGGCBCAYAAYCTSTGYTTYA TTRATBGCVRVCTGYTGYTTRTC GARGCBTGGTGTHWVGATCA YT[+C]YARR[+T]CRAANACGTT GGVTAYAACRTNKSMAAYTTTGA	Sense Antisense Sense Antisense Sense Antisense	Touchdown-PCR 5 min at 95°C 15 cycles (94°C for 1 min, 63°C with decrement by 1°C per cycle for 1 min, 72°C for 2 min) 35 cycles (94°C for 1 min, 48°C for 1 min, 72°C for 2 min) 72°C for 10 min	Chapter Three

		Gam maF WD1 Gam maR EV1	GGRTASAGGCTRGCAAA RTC			

5.5. Results

5.5.1. Overview of viruses identified by Metagenomics Sequencing

Illumina sequencing resulted in 74,169,258 reads for pool 2 and 71,170,820 reads for pool 3. Pool 1 failed the initial quality control check (too low concentration), hence was not sequenced. The reads were filtered, and *de novo* assembled in SPAdes to generate 474,402 and 394,060 contigs for pools 2 and 3 respectively. Paired-end reads (after trimming) were merged into 62,832,276 reads for pool 2 and 63,281,824 reads for pool 3. DIAMOND BLASTX analyses (of the merged reads) produced varying proportions of reads from viruses (79.8%), bacteria (19.5 %), eukaryotes (0.43%) and archaea (0.02 %). More than 90% of the identified viruses (including bacteriophages) were DNA viruses, and less than 10% were RNA viruses of the realm Riboviria. A small proportion of vertebrate (marsupial) viruses (0.047%) was identified compared to non-vertebrate viruses including bacteriophages (85.3%), plant viruses (14.6%), insect viruses (0.018%) and eukaryotic viruses (0.021%). Viral families of the order Caudovirales (tailed bacteriophages) were the most dominant DNA viral groups detected (>50% of total viral abundance) while other viral families were present at much lower abundance including *Adenoviridae, Parvoviridae, Polyomaviridae, Picobirnaviridae, Circoviridae* (marsupial-associated viruses); *Microviridae, Inoviridae,*

Tectiviridae (non-tailed bacteriophages); *Tymoviridae, Virgaviridae, Geminiviridae* (plant viruses); *Iridoviridae, Dicistroviridae , Poxviridae* (insect viruses); *Genomovirdae, Mimiviridae, Phycodnaviridae*, and *Marseilleviridae*, (eukaryotic viruses). The relative abundance of these viral families within each host group is depicted in Figures 5.1 and 5.2. More information on the abundance of each viral family is provided in Appendix 4: Supplementary Table 5.1. Also, additional information on all marsupial related viruses identified in this study including information on contig name, length and sequencing coverage depth and breadth is presented in Supplementary Table 5.2.

5.5.2. Detection and characterization of viruses from agile wallaby fecal samples

5.5.2.1. Marsupial related viruses

Adenoviridae

The partial or complete protein coding genomic sequences of an adenovirus including the hexon, E4, DNA binding protein, DNA polymerase, fiber, 100k, pDNA terminal protein, and IVa2 genes were identified in the libraries of pool 2 and 3. Most of the identified sequences contained reading frameshifts (insertions/deletions) based on BLAST similarity search results, and subsequent sequence editing produced short sequences (less than 150 bp) that were not suitable for phylogenetic analysis, hence they were discarded. A translated hexon protein sequence showed greatest similarity (91%) with the *Possum atadenovirus A*. The other translated protein sequences showed relatively low identity to bovine, tern and ovine adenoviruses (43 to 68%). Due to the availability of a complete hexon protein sequence of the *Possum atadenovirus A* that would allow adequate comparison, coupled with the high similarity of this region to the sequences identified in this study, I chose the hexon protein

sequence (140 aa; after editing to remove frame shifts) for further analysis. Phylogenetic analysis based on hexon protein with representative species in the genus *Atadenovirus* confirmed that the new adenovirus belonged to this genus, and distinctly clustered with the *Possum atadenovirus A* (Figure 5.3a). The virus has been provisionally named agile wallaby atadenovirus 1 (AwAdV-1). A consensus PCR assay (Table 5.1) that amplifies a short region (320 bp) of the polymerase gene of adenovirus was used to confirm the presence of an atadenovirus in pools 2 and 3.

The partial genome sequence of AwAdV-1 was recovered and found to be 31,512 bp in length, with a coverage depth of 22x (sequence coverage of 4756-fold) and 34.4% G+C content. The genome was predicted to contain 32 ORFs with an orientation typical of atadenoviruses. Of the 32 ORFs, 26 were annotated with varying similarities to the coding genes of other atadenoviruses (Tables 5.2). The IVa2, penton base protein, pX and hexon genes showed the highest amino acid identity (71-87%) to the reference mammalian atadenoviruses (Table 5.2). The AwAdV-1 genome possesses frame shifts due to insertions and deletions in most of the genes except IVa2, pX, pVI, pVIII and U-exon genes. Two fiber genes namely, fiber and IV-1 (homologous to the fiber 2 gene in lizard adenovirus 2) were present in the genome of AWAdV-1 instead of the single long fibre gene in mammalian atadenoviruses. The inverted terminal repeats (ITR) were missing at both the 5′ and 3′ ends. Genus demarcation based on phylogenetic analysis of the amino acid of DNA polymerase further confirmed that this virus is a member of the genus *Atadenovirus* (Appendix 4: Supplementary Figure 5.1).

Parvoviridae

Two new species of parvovirus belonging to the sub-family *Hamaparvovirinae* and the genus *Chaphamaparvovirus* were identified in this study. The detected partial protein sequences (72-102 aa) shared 58% and 82% identity with the capsid protein (NS1) of Tasmanian devil-associated chapparvovirus 3 and Tasmanian devil-associated chapparvovirus 1 respectively. The new viral species were tentatively named agile wallaby chaphamaparvovirus 1 and 2. Phylogenetic analysis confirms clustering of these new viruses with the Tasmanian devil-associated chapparvoviruses (Figure 5.3b). PCR assay using specific primers (Table 5.1) derived from the nucleotide sequence of agile wallaby chaphamaparvovirus 1, detected the virus in five individual samples from pool 2 and three samples from pool 3. Genus demarcation based on phylogenetic analysis of the amino acid of NS1 further confirmed that these viruses are members of the genus *Chaphamaparvovirus* (Appendix 4: Supplementary Figure 5.2).

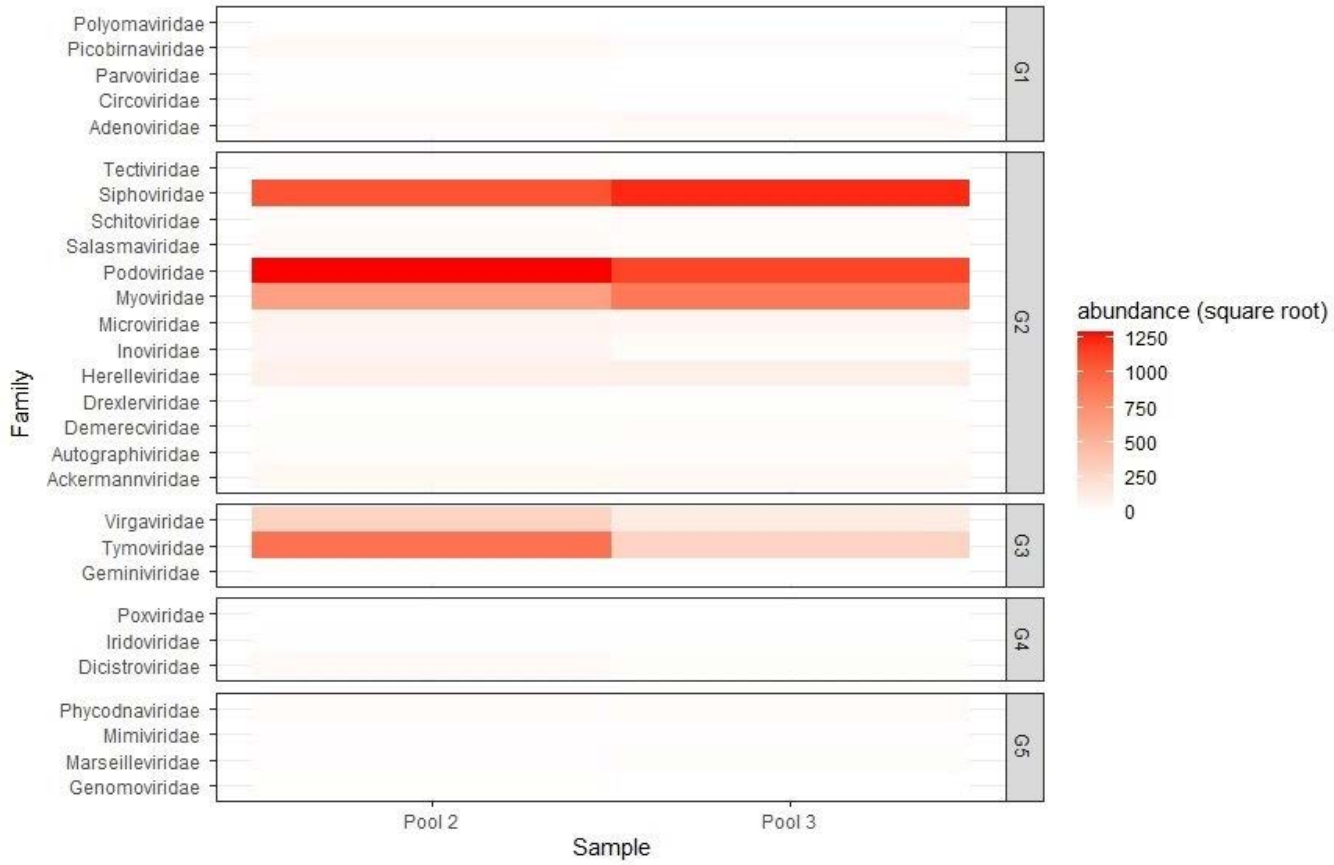


Figure 5. 1. Overview of agile wallaby fecal viruses identified by metagenomics in a heatmap diagram, where intensity of colour indicates relative abundance of viral families (G1 = marsupial-associated viruses, G2 = bacteriophages, G3 = plant viruses, G4 = insect viruses, G5 = eukaryotic viruses). BLAST analysis and taxonomic assignment were performed on merged reads using DIAMOND BLASTX and Krona respectively.

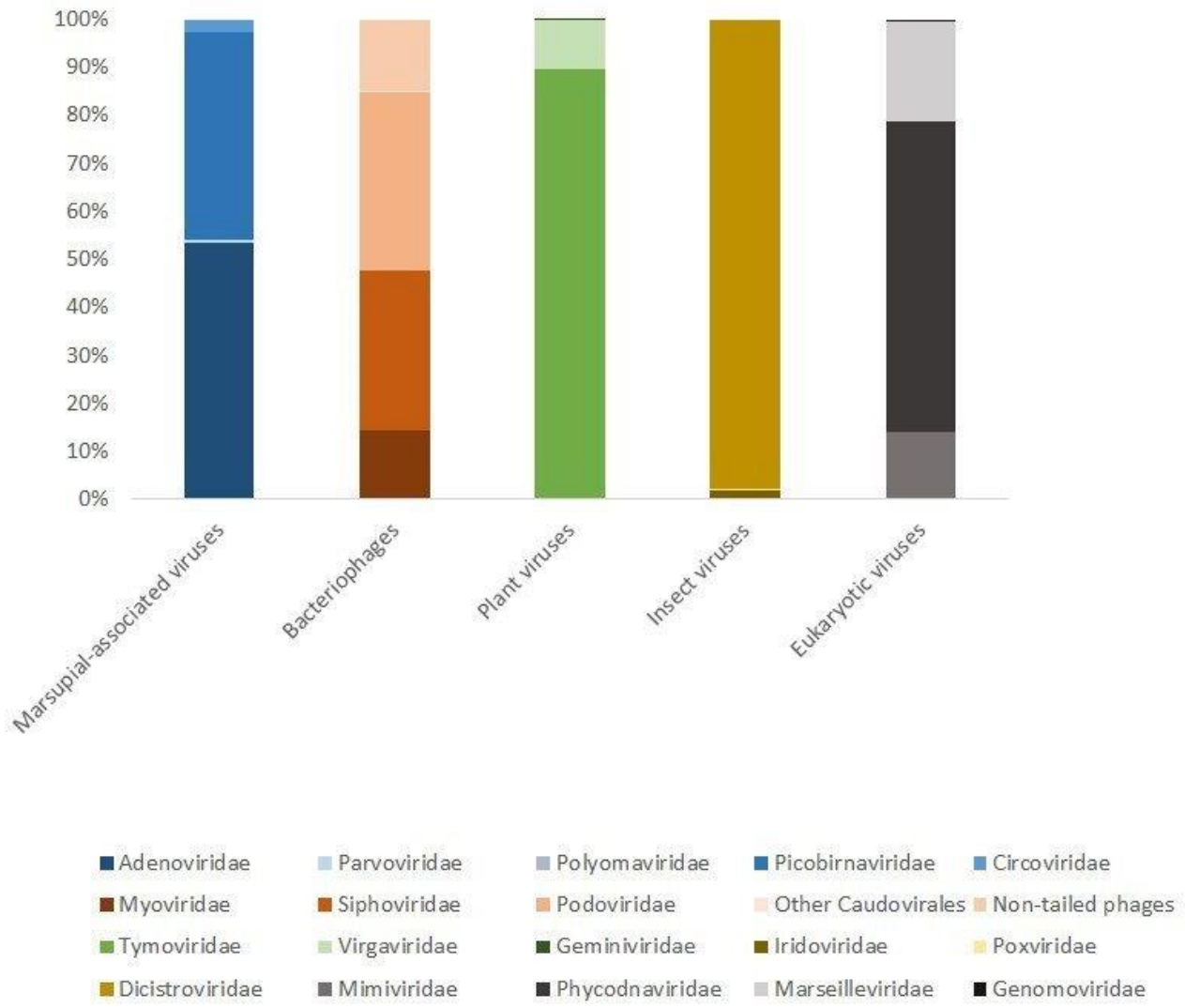


Figure 5. 2. The composition and relative abundance of fecal viruses within each viral host group.

Polyomaviridae

A novel virus, provisionally named agile wallaby polyomavirus 1 was identified in the metagenomics library of pool 3. The identified short protein fragment (246 bp) that encodes the major capsid protein shares 67% identity with the Tasmanian devil-associated polyomavirus 1. PCR (Table 5.1) successfully amplified a 255 bp region of the major capsid gene in two (one from each pool) out of the 11 individual samples that made up pools 2 and 3. Sanger sequencing of the two PCR products returned similar sequences (96%), and

phylogenetic analysis based on the major capsid protein revealed another novel virus provisionally named agile wallaby polyomavirus 2. The two novel polyomaviruses form a distinct clade with the Tasmanian devil-associated polyomavirus 1 (Figure 5.3c).

Picobirnaviridae

Picobirnavirus nucleotide sequences encoding the RNA dependent RNA polymerase (RdRp; 82-222 aa) were detected in the libraries of pools 2 and 3. The sequences were tentatively named agile wallaby-associated picobirnavirus 1 to 9. The novel viruses showed varying similarity (46% to 89%) to the picobirnaviruses isolated from sheep, porcine, rhesus macaque, Tasmanian devils, and marmots. Phylogenetic analysis based on the RdRp protein showed that these viruses were highly diverse and clustered widely across the phylogeny (Figure 5.3d).

The complete large genome segment 1 of agile wallaby-associated picobirnavirus was recovered and found to be 2482 bp in size, with a coverage depth of 20x and 34.85% G+C content. This genome segment had two ORFs encoding the capsid protein (1440 bp) and an unknown protein (555 bp). Amino acid sequence comparison of the capsid protein showed varying similarities with the equine picobirnavirus (AKN50626; 26%), human picobirnavirus (YP_239360; 23%), marmot picobirnavirus (AVX29477; 29%), and porcine picobirnavirus (ASM93458; 25%). The smaller genome segment 2 (1712 bp; 40.89% G+C content) of the agile wallaby-associated picobirnavirus 1 contained one ORF that encodes the RdRp (1629 bp), with a coverage depth of 65x. The RdRp amino acid showed the highest similarity (58-59%) with the prototype sequences of genogroup I (YP_239361 and AAG53583) and less similarity (23%) with the protype sequence of genogroup II (AAG53584). The RdRp gene possesses frameshifts due to insertions and deletion. Phylogenetic analysis based on the

amino acid sequence of RdRp showed that the agile wallaby-associated picobirnavirus 1 belongs to genogroup I (Appendix 4: Supplementary Figure 5.3).

Circoviridae

A short fragment of the nucleotide sequence (244 bp) that encodes the Rep protein of a circovirus species, was detected in one of the metagenomics libraries (Pool 2). Phylogenetic analysis based on the Rep protein showed that the novel circovirus clustered with a circovirus recovered from human metagenome (Figure 5.3e) and shares a high protein similarity (85%) with this virus. An attempt to verify the presence of circoviruses in individual samples by PCR resulted in the detection of a novel cyclovirus (360 bp). This virus shares the highest nucleotide similarity (87%) with a rodent cyclovirus. These novel viruses may be more related to other host species other than marsupials based on their sequence identity and phylogenetic clustering; however, further characterization study would be required to conclusively associate these viruses to a specific host.

Herpesviridae

Herpesvirus could not be detected in the pooled samples by neither metagenomics nor consensus PCR approaches. However, two (from pools 1 and 2) out of the 16 individual samples screened for HV were positive by consensus PCR assays. BLASTN analysis of the sequences obtained by Sanger method showed a high similarity (96%) with a previously identified HV, Macropodid herpesvirus 3 (MaHV-3). Thus, the sequences were assigned to MaHV-3 and no further analysis was required.

Other viruses: bacteriophage, plant, insect viruses and eukaryotic viruses

A considerable proportion of viruses identified in this study was assigned to viruses that infect bacteria and plants (Figure 5.1). Bacteriophages of the order *Caudovirales* were the most dominant sequences, accounting for >50% of all the viral reads. The insect viruses identified in this study belong to the families *Iridoviridae*, *Dicistroviridae*, and *Poxviridae* (genus *Betaentomopoxvirus*). Sequences related to the viruses of plants, algae and fungi were identified in both libraries and included members of the families *Tymoviridae*, *Virgaviridae*, *Geminiviridae*, *Genomoviridae*, *Mimiviridae*, and *Phycodnaviridae*.

Table 5. 2. Sequence comparison with the genomes of mammalian atadenoviruses.

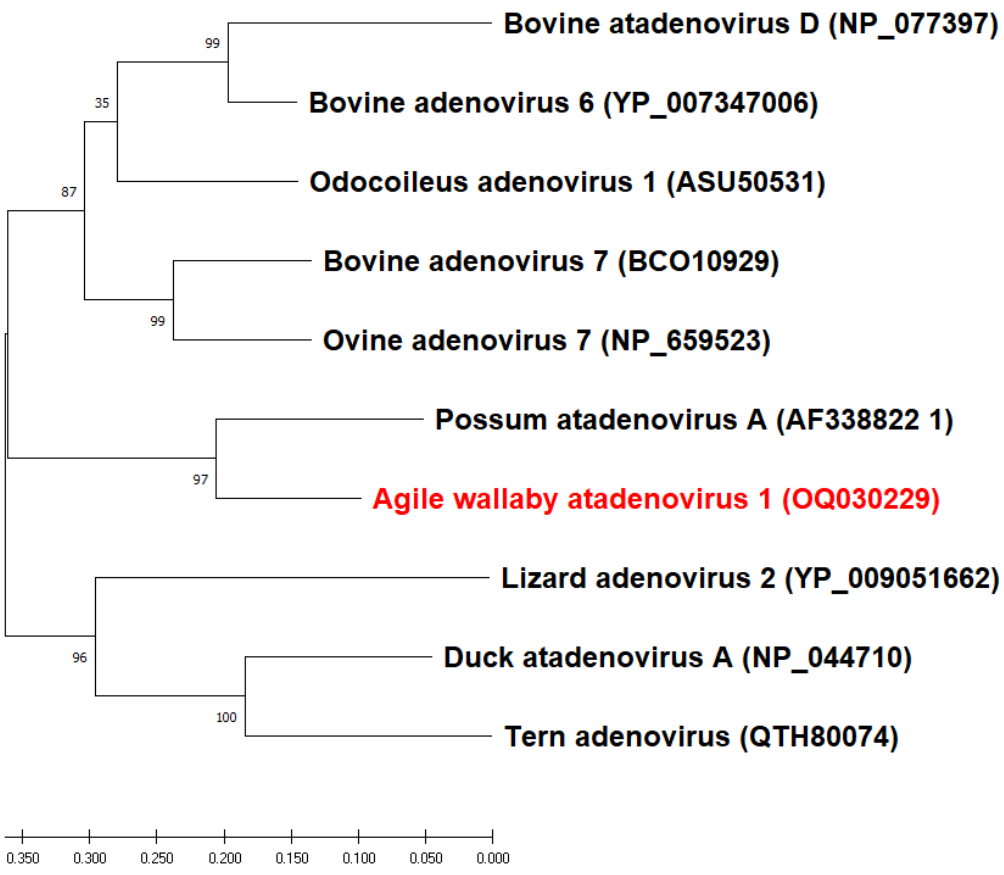
		Agile wallaby atadenovirus 1		Ovine adenovirus (NC_004037)			Bovine adenovirus D (NC_002685)			Bovine adenovirus E (NC_020074)			Odocoileus adenovirus 1 (NC_035619)		
Name	Description (putative)	Length (nt)	Length (aa)	Length (nt)	Length (aa)	aa Identity (%)	Length (nt)	length (aa)	aa Identity (%)	Length (nt)	Length (aa)	aa Identity (%)	Length (nt)	Length (aa)	aa Identity (%)
orf0001	hypothetical protein	255	85	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf0002	hypothetical protein	402	134	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00003	p32K	1032	344	861	286	39	819	272	45	891	296	40	930	309	35
orf00004	LH1	396	132	363	120	29	378	125	29	378	125	26	393	130	25
orf00005	E1B 55K	1293	431	1149	382	43	1161	386	42	1152	383	45	1146	381	43
orf00006	IVa2	903	301	984	327	75	966	321	74	966	321	72	1209	402	72
orf00007	Pol	3243	1081	3216	1071	58	3222	1073	58	3222	1073	57	3228	1075	58
orf00008	pTP	1773	591	1788	595	50	1803	600	51	1803	600	51	1800	599	51
orf00009	52K	987	329	1008	335	62	1059	343	62	1035	344	60	1014	337	58

orf00010	pIIIa	1767	589	1707	568	53	1722	573	54	1551	516	54	1749	582	53
orf00011	penton base protein	1347	449	1359	452	68	1353	450	67	1359	452	68	1353	450	71
orf00012	pVII	345	115	336	111	54	360	119	57	357	118	56	354	117	51
orf00013	pX	102	34	216	71	87	216	71	81	219	72	0	No data	No data	No data
orf00014	pVI	669	223	666	221	54	603	200	57	612	203	56	678	225	54
orf00015	Hexon	2730	910	2736	911	74	2733	910	72	2733	910	76	2733	910	74
orf00016	23K endoprotease	606	202	606	201	59	606	201	60	606	201	61	606	201	60
orf00018	DNA binding protein	999	333	1149	382	56	1143	380	58	1140	379	56	1158	385	57
orf00020	100K	2055	685	1878	625	54	1887	628	56	1887	628	54	1914	637	55
orf00021	33k	512	170	402	133	39	405	134	65	408	135	39	414	137	38
orf00022	pVIII	780	260	654	217	44	669	222	44	672	223	45	681	226	49
Orf00023	U-exon	165	55	177	58	35	165	54	54	165	54	54	165	54	54

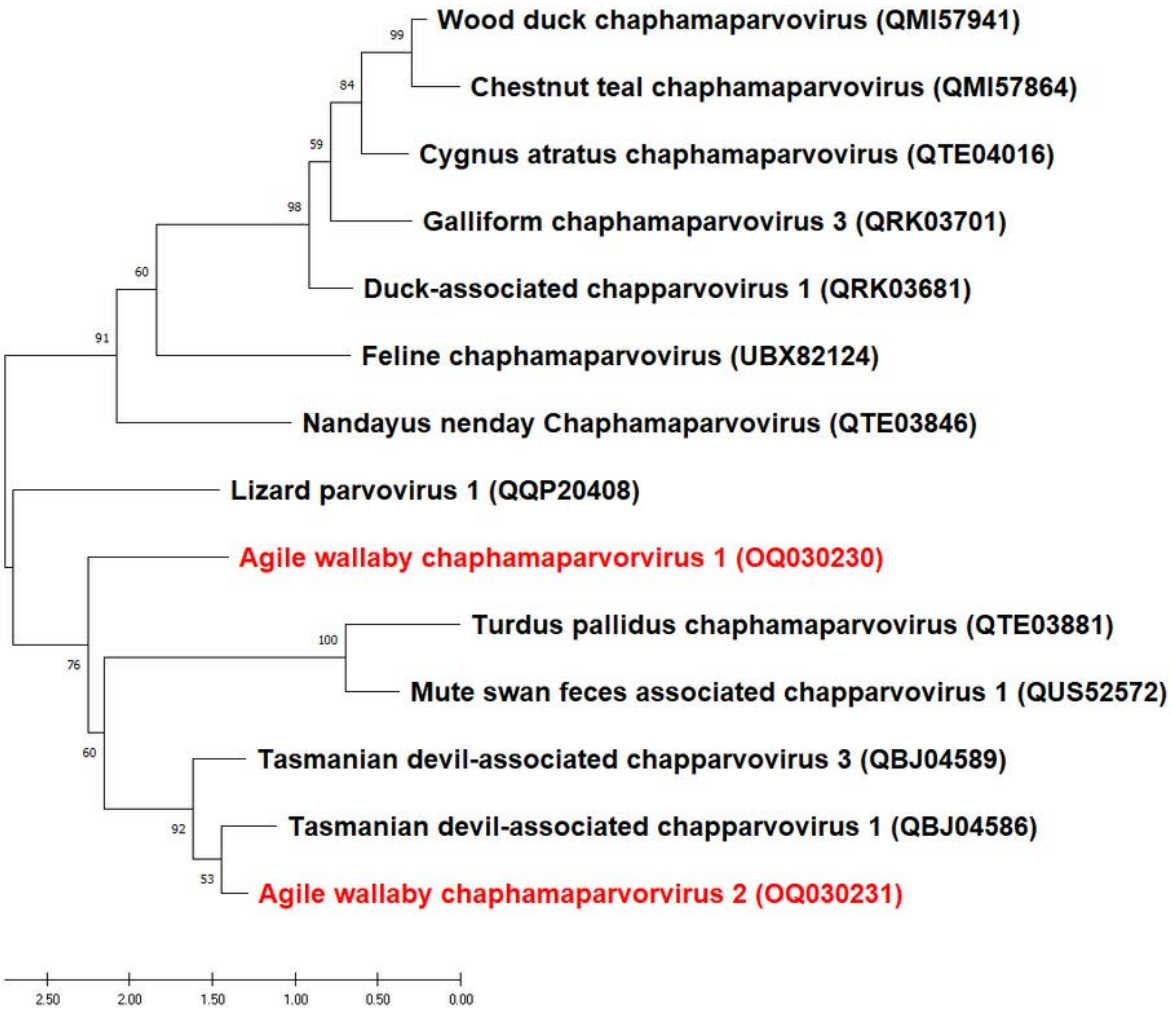
orf00024	Fiber	891	297	1632	543	36	1608	535	36	1332	443	43	1422	473	29
orf00025	IV-1	1554	518	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00026	E4.3	600	200	714	237	34	654	217	38	657	218	38	705	234	33
orf00027	E4.2	678	226	663	220	40	678	219	42	660	219	42	678	219	39
orf00028	E4.1	441	147	429	142	36	429	142	37	429	142	38	429	142	30
orf00029	RH0	381	127	No data	No data	No data	564	187	45	No data	No data	No data	No data	No data	No data
orf00030	RH5	606	202	597	198	26	624	207	27	651	216	24	624	207	34
orf00031	hypothetical protein	372	124	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00032	hypothetical protein	201	67	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00033	hypothetical protein	366	122	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00034	hypothetical protein	372	124	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data

nt: nucleotide; aa: amino acids.

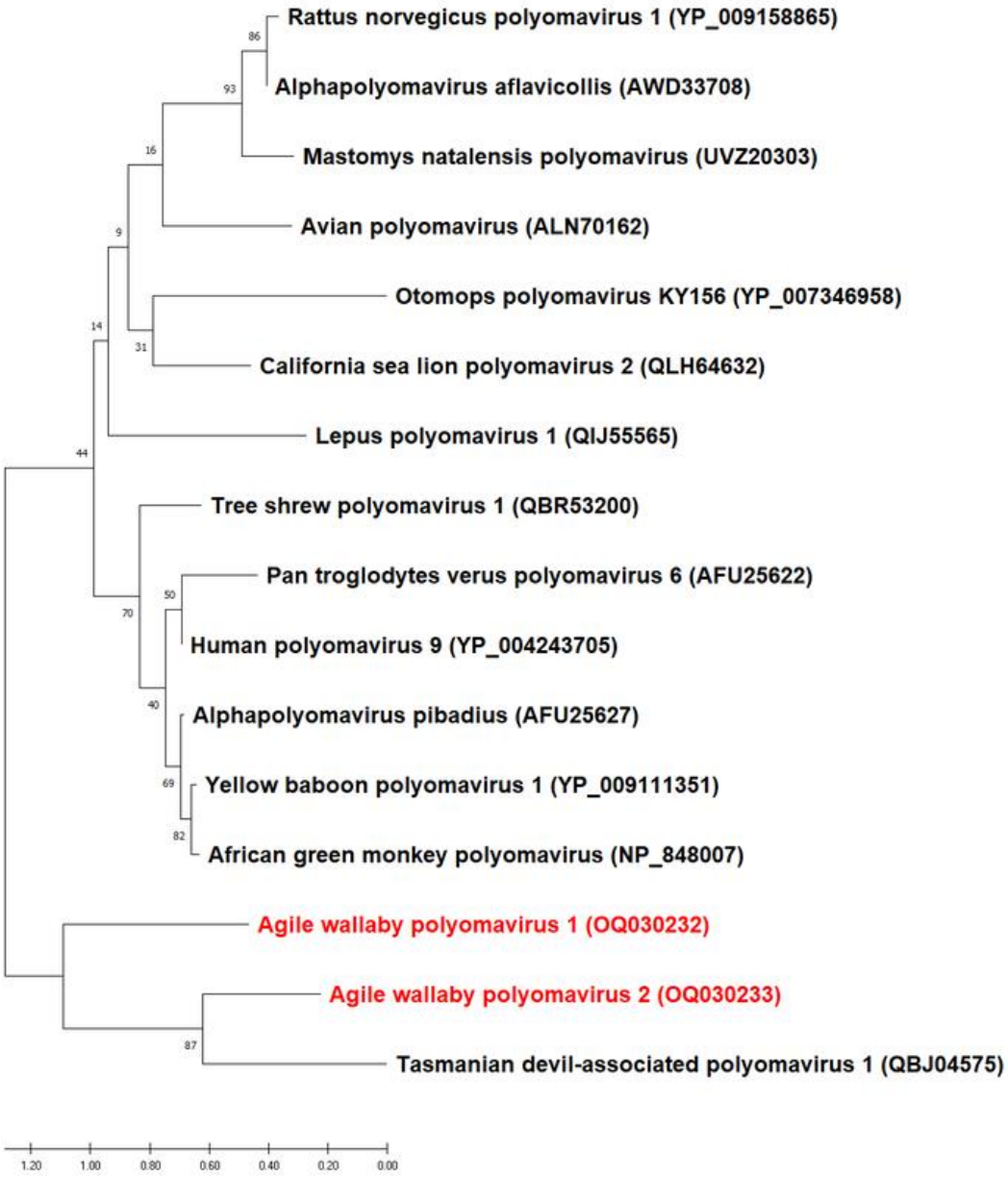
a. Adenoviridae



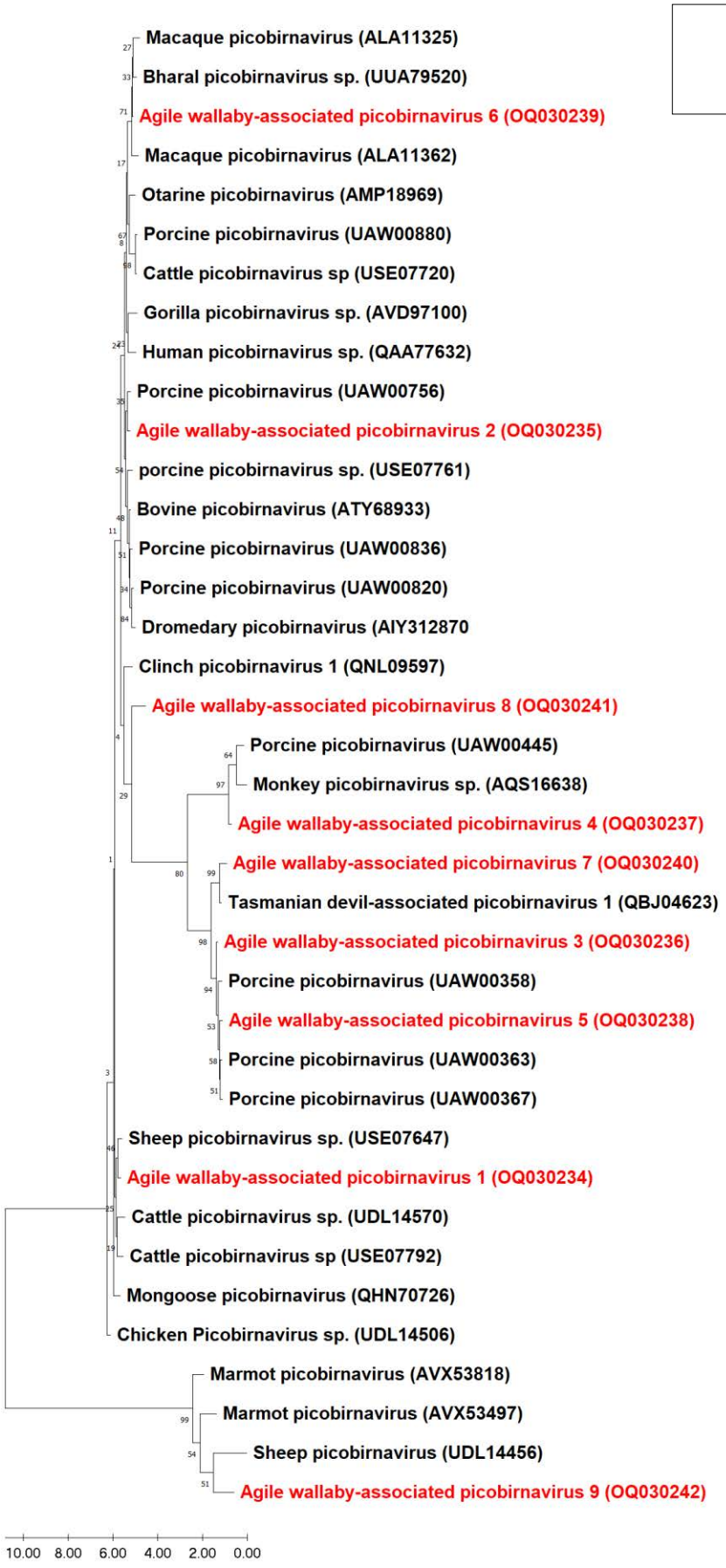
b. Parvoviridae



c. Polyomaviridae



d. Picobirnaviridae



e. Circoviridae

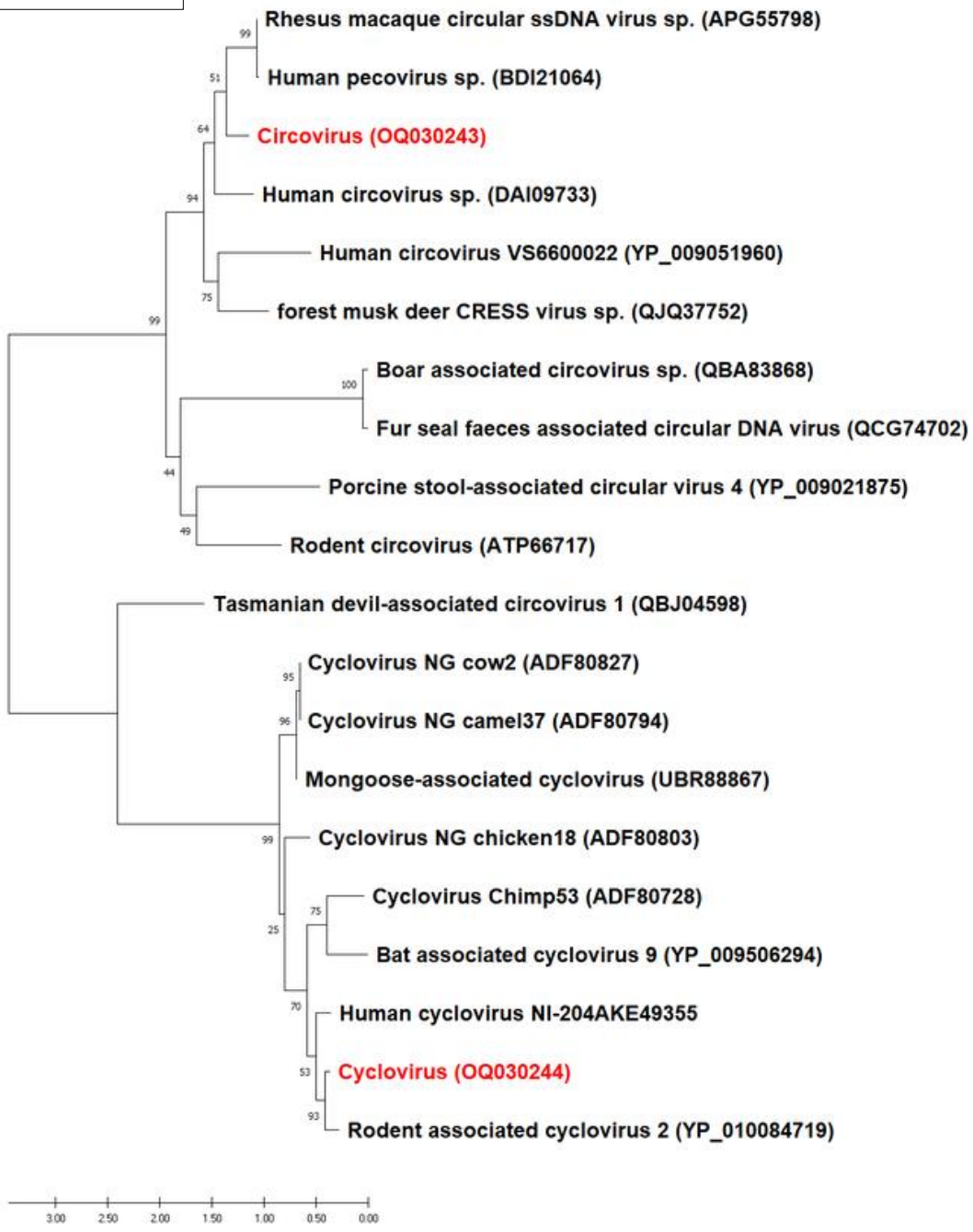


Figure 5. 3. Phylogenetic analysis of the viruses identified in the fecal samples of agile wallabies. Novel viruses are shown in red. (a) Agile wallaby atadenovirus 1 based on the amino acid sequences of hexon protein. (b) Agile wallaby chaphamaparvovirus 1 and 2 based on the amino acid sequences of capsid protein. (c) Agile wallaby polyomavirus 1 and 2 based

on the amino acid sequences of major capsid protein. (d) Agile wallaby-associated picobirnavirus 1 to 9 based on the amino acid sequences of RNA dependent RNA polymerase protein. (e) Circovirus and cyclovirus based on the amino acid sequences of the Rep protein. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.

5.6. Discussion

Metagenomics and molecular techniques were used in this study to identify a wide variety of viruses including vertebrate and non-vertebrate viruses in agile wallaby fecal samples. Overall, I detected 14 novel agile wallaby viruses and one known marsupial herpesvirus (MaHV-3). These viruses belong to viral families that include viruses of known pathogenic significance. For instance, parvoviruses have been associated with severe gastroenteritis with a high fatality rate in carnivores (Gainor et al., 2021; Watanabe et al., 2020). Similarly, herpesviruses were incriminated as the cause of different outbreaks of fatal disease in wallabies (Callinan & Kefford, 1981; Finnie et al., 1976; Wilks et al., 1981). However, the pathogenic potential of the novel viruses identified in this study is unclear.

Adenoviruses are non-enveloped, icosahedral viruses with linear, unsegmented double-stranded DNA genomes (Madarame et al., 2019). The genomes of adenoviruses range in size from 26 to 48 kb, and commonly contain between 22 and 40 genes (Davison et al., 2003; Madarame et al., 2019). On the other hand, picobirnaviruses are non-enveloped viruses containing two linear dsRNA genome segments (Li et al., 2015). The larger segment1 (2200-2700 bp) possesses a capsid gene and one or two ORFs that encode unknown proteins; the smaller segment2 (1200-1900 bp) possesses the RdRp encoding gene (Li et al., 2015). In this study, the near-complete genomes of AwAdV-1 and agile wallaby associated picobirnavirus 1 were recovered from the metagenome of agile wallabies. These genomes possessed taxa-

specific characteristics in terms of genome composition and arrangement. Over the years, the characterization of many novel viruses was limited by the lack of specific tissue or cell cultures for viral isolation (Böszörményi et al., 2020; Sykes & Rankin, 2014). However, advances in molecular approaches including metagenomics have made it possible to identify and characterize viral sequences without prior isolation (Böszörményi et al., 2020; Roux et al., 2021; N. Sandybayev et al., 2022). Moreso the International Committee on Taxonomy of Viruses (ICTV) has stated that viral sequences identified from metagenomic data can, and should be accepted as official virus species (Simmonds et al., 2017). The near-complete genomes of marsupial adenovirus and picobirnavirus assembled in this study would enrich the diversity of knowledge about marsupial viruses, and can be useful for developing diagnostics, therapeutics, and vaccines.

The frameshifts identified in some of the genes were largely due to sequencing errors and an attempt to correct them by running SPAdes on a "careful" mode did not produce the desired outcome. Despite significant improvements in sequencing technologies, standard sequencing platforms are still at risk of random and systematic errors (approximately 0.1–1%), and computational error correction algorithms have limited accuracy (Mitchell et al., 2020). Although acceptable for qualitative detection (presence or absence of viruses following sequence editing), sequencing error is undesirable in certain clinical applications such as detection and quantification of sequence variants, endogenous viral elements, and measurable residual disease (MRD) (Crowgey et al., 2020; Patkar et al., 2021). The recent development of error corrected NGS (ecNGS) has the potential to revolutionize the field of genomics and metagenomics. ecNGC employs consensus sequencing of unique molecular identifier (UMI) tagged DNA duplex in conjunction with bioinformatics to remove sequencing errors from reads, resulting in error rates of less than 0.000001% (Marchetti et al., 2023; Mitchell et al., 2020; Salk et al., 2018).

Phylogenetic analysis showed a close relationship and evolutionary history of some novel viruses including adenoviruses, parvoviruses, and polyomaviruses, with marsupial viruses. This observation indicates that marsupial viruses form specific lineages that are distinct from the viruses of placental mammals (Eutherians). However, clustering with both marsupial and eutherian viruses (with low bootstrap support) was observed for picobirnaviruses. This non-specific clustering could be due to the high genetic diversity of picobirnaviruses (Duraisamy et al., 2018; Ng et al., 2014). The clustering patterns observed in this study support existing evidence that mammalian viruses often co-evolve with their hosts (Harvey & Holmes, 2022; Kaján et al., 2020; Simmonds et al., 2019). Interestingly, one of the novel circoviruses identified in this study clustered with the human circovirus with high nucleotide similarity. Previous studies have identified many novel circoviruses in NGS data including the hepatitis associated human circovirus 1 (HCirV-1) (Pérot et al., 2023; Porter et al., 2021; Rosario et al., 2017). However, circoviruses have been implicated as major contaminants of laboratory reagents, resulting in incorrect inferences on host associations (Porter et al., 2021). To further investigate and possibly rule out contamination, we conducted PCR on the pooled libraries, individual samples and a no template control (NTC). A closely related novel virus (cyclovirus) was detected in the individual samples and no positive band was observed for the NTC. These findings imply that the novel circoviruses are likely true host-associated viruses and not viral contaminants. Availability of full-length sequences for these viruses would provide reliable topologies that allow host associations to be accurately predicted. Therefore, further studies should be conducted to elucidate the host of these novel viruses and assess their potential health impact on putative hosts.

Bacteriophages dominated the fecal viral content of agile wallabies with a viral read assignment of 85.3%. Similarly, in a previous study using virion-enriched metagenomics, bacteriophage constituted close to 99% of all the viral reads obtained from the feces of

Tasmanian devil (Chong et al., 2019). This high abundance is in line with other studies, where bacteriophages were found to form the bulk of the viral component of the gut microbiota (virobiota) (Cao et al., 2022; De Paepe et al., 2014). Bacteriophages are known to interact with the host immune system by enhancing innate immunity and clearing potentially harmful bacteria (Popescu et al., 2021; Van Belleghem et al., 2018). While the specific role of bacteriophages in the health of agile wallabies is yet unknown, I have provided baseline metagenomics data that can be explored in future research. Conversely, marsupial-related viruses were found at low abundance in this study, and this could imply a lack of active infection or low viral shedding. However, the detection of certain parenteral viruses (e.g., hepadnaviruses) in feces could be as result of contamination with gastro-intestinal fluid, urine or blood and have no relevance in relation to fecal viral abundance, due to their sporadic occurrence in host feces (Komatsu et al., 2015; Zheng et al., 2015). The fecal viral load of enteric viruses could serve as an important epidemiological indicator during disease outbreaks as high viral shedding indicates active viral replication and an increased risk of disease transmission within the affected populations (Kopel et al., 2020; Martens et al., 2020; Nobach et al., 2015; Santiana et al., 2018). However, to use fecal viral load data of enteric viruses as an important epidemiological indication during disease outbreaks, future studies should provide baseline data about the seasonal variation of these enteric viruses in agile wallabies. This could be in the form of longitudinal sampling efforts that span different reproductive states of the wallaby colony as viral abundance tends to alter when newborn and naïve animals are present.

In this study, viruses that infect plants, insects and other eukaryotes (fungi, amoeba and algae) were also isolated from the feces of agile wallaby, indicating the ingestion of food containing these viruses. While it is unlikely that food borne viruses will replicate within the wallaby host, there is a risk of wallabies acting as viral vectors between different plant

populations, be they wild or cultured. This is another example of the potential for wildlife to influence pathogen circulation in the One Health nexus.

Generally, metagenomics mostly detected DNA viruses in the feces of agile wallabies. This skewness toward DNA viruses was also observed in previous studies that used a similar approach (Duarte et al., 2019; Ramírez-Martínez et al., 2018). In another study, a relatively good proportion of RNA (5 to 97.51%) and DNA (2.49 to 95.54%) viruses were identified when both metagenomics and metatranscriptomics were complementarily used to characterize the fecal viral diversity of Tasmanian devils. However, both approaches failed to detect all of the viruses, especially those present in low amount (Chong et al., 2019). Likewise, metagenomics and consensus PCR could not detect MAHV-3 in both pooled samples; however, the virus was detected by consensus PCR in two individual samples indicating a possible viral dilution (due to sample pooling) below the detection limit of both assays. These findings highlight the need to further optimize and integrate various approaches including metagenomics, metatranscriptomics and molecular techniques in order to obtain a comprehensive profile of fecal viral diversity.

I have shown in this study that non-invasive fecal sampling can be a vital tool in studying the fecal viral diversity of free-ranging agile wallabies. The use of non-invasive sampling in viral discovery studies and monitoring of disease emergence is gaining significance especially where endangered, invasive, or elusive wildlife species are investigated (Sanyal et al., 2022). This approach is less labor-intensive, more cost-effective, rapid, and minimizes the disruption of already fragile habitats (Sanyal et al., 2022). Traditional sampling methods can provide better insights into the epidemiological characteristics of viruses in wildlife populations as well as enable adequate assessment of diseased populations. However, the two approaches should be combined (whenever possible) such that a smaller number of wild animals are

invasively sampled, and the small sample size is made up for by non-invasively collecting more samples from a given population.

Herein, I have provided baseline information on the fecal viruses of free-ranging agile wallabies using a complementary approach (metagenomics and PCR). This approach, if well consolidated, can become an invaluable tool for monitoring the emergence of potential pathogenic viruses, and can ultimately contribute to the wildlife management plans of threatened marsupial species. To better understand the pathogenicity and zoonotic potential of novel viruses, efforts should be tailored towards the development of specific tissue or cell cultures for viral characterisation. Nonetheless, the viruses of agile wallaby identified in this study could be potential candidates for future research on vaccinology, diagnostics and therapeutics in the event of disease outbreaks caused by any of these or closely related viruses. It is interesting that the small sample size used in this study generated such a large amount of data on the fecal viral diversity of free-ranging agile wallabies. In future research, the study sample size can be increased for epidemiological surveillance and evaluation of potential health impact of viral pathogens on agile wallabies. Also, periodic cohort studies to determine disease persistence and risk factors associated with potential disease outbreaks could benefit wildlife conservation efforts in the future.

5.7. Data availability

The generated raw sequence reads from this study are available in NCBI SRA database under BioProject number PRJNA907146. All the novel sequences and genomes generated in this study have been deposited in GenBank under the accession numbers OQ030229 to OQ030244, OR030846, OR030847, and OQ792214.

5.9. Chapter Summary

The agile wallaby (*Notamacropus agilis*) is one of the most abundant marsupial species in northern Queensland and a competent host for the zoonotic Ross River virus. Despite their increased proximity and interactions with humans, little is known about the viruses carried by these animals, and whether any are of conservation or zoonotic importance. Metagenomics and molecular techniques were used in a complementary manner to identify and characterize novel viruses in the fecal samples of free-ranging agile wallabies. We detected a variety of novel marsupial-related viral species including agile wallaby atadenovirus 1, agile wallaby chaphamaparvovirus 1-2, agile wallaby polyomavirus 1-2, agile wallaby associated picobirnavirus 1-9, and a known macropod gammaherpesvirus 3. Phylogenetic analyses indicate that most of these novel viruses would have co-evolved with their hosts (agile wallabies). Additionally, non-marsupial viruses that infect bacteria (phages), plants, insects, and other eukaryotes were identified. This study highlighted the utility of non-invasive sampling as well as the integration of broad-based molecular assays (consensus PCR and next generation sequencing) for monitoring the emergence of potential pathogenic viruses in wildlife species. Furthermore, the novel marsupial viruses identified in this study will enrich the diversity of knowledge about marsupial viruses and may be useful for developing diagnostics and vaccines.

5.10. My contributions to this chapter

- I, in collaboration with my advisors conceptualize and designed this work.
- I collected samples with the support of my primary supervisor.
- I conducted laboratory analysis including viral enrichment, nucleic acid extraction, cDNA synthesis and PCR.

- I conducted all the bioinformatic analysis in this chapter including sequence mining, alignments, primer design, phylogenetic analysis, and analysis of NGS data (genomics and metagenomics).
- I submitted the novel sequences to GenBank.
- I collated data tables and figures under the advice of my supervisors.
- I drafted the chapter and edited it as advised by my supervisors.
- I managed the process of journal submission and review.

Chapter Six: General Discussion

The discovery of viruses and the infections they cause commenced in the late 19th century (Taylor, 2014). The first viral discovery study was conducted in 1892 when Dmitri Ivanovsky showed that filtered sap from diseased tobacco plant remained infectious to healthy tobacco plants (Lecoq, 2001; Taylor, 2014). This filtered infectious agent was coined "virus" in 1898 by Martinus Beijerinck when he observed that the infectious agent (now known as tobacco mosaic virus) could only multiply in actively dividing cells (Lecoq, 2001). In the same year (1898), Friedrich Loeffler and Paul Frosch conducted the filtration experiment and discovered the first animal virus as the cause of foot-and-mouth disease (Brown, 2003; Fenner, 2008). Despite the overwhelming evidence linking microbes to diseases, the germ theory of disease was still debatable until late in the 19th century when Friedrich Loeffler (a protégé of Robert Koch) formulated the "Koch's postulates" (Walker et al., 2006). This theory was outlined to establish an association between microorganisms and disease, and it states that (1) organism must be detected in diseased tissue in their attributable form and organisation, (2) this organism must be isolated and grown in pure culture, (3) the pure culture must be demonstrated to experimentally induce the disease and (4) the organism should be reisolated from the experimentally induced subject (Walker et al., 2006). However, several attempts to apply these postulates to viruses were unsuccessful because viruses could not be seen nor isolated in culture at that time (Walker et al., 2006). The discovery and partial characterisation of viruses by traditional approaches including filterability and ultra-visibility with optical microscope continued into the 20th century until the invention of electron microscope in 1931 by Ernst Ruska and Max Knoll (Freundlich, 1963; Mettenleiter, 2017). The introduction of electron microscopy revolutionised the understanding of some viral properties including their form (particles), structure, size, and composition (proteins and nucleic acids) (Burnet & Stanley, 2013; Kay, 1986). In recent decades, the advent and

development of molecular techniques including universal PCR and next generation sequencing have led to the rapid and accurate identification of a variety of new viruses in a hitherto improved depth and sensitivity (Cassedy et al., 2021; Epstein & Anthony, 2017; Mettenleiter, 2017). The association of emerging novel pathogens with diseases is still challenging, especially for those that are non-culturable (Ramamurthy et al., 2014; Wang, 2020). To characterise the microbial diversity of healthy and diseased animals and cast light on a cause and effect of microbes, a newer postulate, considering modern techniques and age-old dilemmas is called for. The Bradford Hill criteria, first proposed in 1965 by Sir Austin Bradford Hill (Hill, 1965), may offer a viable alternative for inferring causal relationships in instances where microbial cultivation is unattainable. The criteria consist of nine guidelines that can be useful in establishing evidence of a causal relationship between cause and effect (Hill, 1965). Their application in clinical and epidemiological outcomes have been a subject of debate for years (Holger et al., 2011; Rothman & Greenland, 2005). However, some useful propositions on how to use these criteria have been made (Glass et al., 2013; Höfler, 2005; Howick et al., 2009; Potischman & Weed, 1999; Rothman & Greenland, 2005), and one that stands out was made by Potischman and Weed (Potischman & Weed, 1999), who recommended using the criteria as only a guide and not definitive conclusions.

Viral diseases can potentially threaten the conservation of wildlife species and cause major public health concerns (Parrish et al., 2008; Sarker, 2022). In Australia, the impact of viral diseases on the health and wellbeing of wildlife species has not been completely elucidated due to limited virological studies. Herpesviruses are one of the most important causes of wildlife disease in Australia, and clinical diseases from these viruses have mostly been described in captive animals (Smith et al., 2008; Woźniakowski & Samorek-Salamonowicz, 2015). Knowledge on viruses, including herpesviruses, circulating in Australian wildlife is essential to improving current management or conservation plans. The goals of this study

were to design sensitive molecular assays for herpesvirus detection, use these assays as well as other molecular techniques to identify herpesviruses and other potential pathogenic viruses present in wild populations of freshwater turtles and agile wallabies, and propose an effective workflow for viral discovery and disease monitoring in wildlife.

Sensitive tools are needed for the investigation of HVs in wildlife species (Epstein & Anthony, 2017; Okoh et al., 2021). Currently available HV consensus PCR (universal PCR) assays are limited by low or selective sensitivity and require a nested format, which are prone to contamination (Epstein & Anthony, 2017; Prepens et al., 2007). In Chapter 3, these challenges were addressed by the development of a non-nested consensus PCR (STC-PCR) that can sensitively amplify the DNA fragments of alpha-, beta-, and gamma-HVs. The STC-PCR was successfully evaluated with a large collection of human and animal HV clinical samples in two independent laboratories. The STC-PCR was found to have comparable sensitivity with the commonly used universal nested PCR assay (VanDevanter et al., 1996), and showed improved band quality on an electrophoretic gel. Generally, consensus PCR assays use degenerate primers that bind to conserved regions flanking stretches of variable sequences, thus allowing for the "universal" amplification of viral sequences (Epstein & Anthony, 2017). However, the consensus PCR has the potential to miss sequences that show divergence in these conserved regions leading to reduced sensitivity (Epstein & Anthony, 2017). The strength of the STC-PCR is that it targets genes at a lower taxonomic level (subfamily) where sequences are less divergent (more conserved) while retaining its universal applicability. In addition, the STC-PCR is less expensive, more time efficient and less prone to contamination, thus making it a suitable tool for viral discovery. The applicability of the STC-PCR in wildlife viral discovery is however limited by the fact that the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) contains many partial HV genomes or gene fragments that were mostly generated by previous consensus PCR primers. These available gene

fragments correspond to regions (in HV genomes) that are different from those targeted by the STC-PCR primers, thus making sequence comparison (phylogenetic analysis) impossible/difficult. Therefore, the STC-PCR should be used in complementarity with currently available consensus PCR assays until relevant sequences of different HVs are adequately generated for comparison.

In Chapter 4, the STC-PCR developed in Chapter 3 was used in combination with other consensus PCRs to investigate the presence of HV and other suspected lesion causing viruses including AdV, papillomavirus and poxvirus in wild freshwater turtles with cutaneous lesions. In this study, six new HVs and AdVs were identified for the first time in any species of Australian freshwater turtle indicating the effectiveness of the use of consensus PCR assays for viral discovery in wildlife. The occurrence of HVs pose a potential threat to the affected populations of freshwater turtles since HVs are known to form lifelong infections and cause severe diseases in immunosuppressed or naïve populations (Okoh et al., 2021). In reptiles, HVs have been associated with epizootics of epidermal necrosis, pneumonia, and necrotizing oral and upper respiratory inflammation, as well as cutaneous papillary tumour (fibropapillomatosis) that could lead to death in green sea turtles (Okoh et al., 2021; Ossiboff et al., 2015). Similarly, cutaneous lesions and fatal systemic lesions caused by HVs have been described in freshwater turtles (Ossiboff et al., 2015). AdVs in chelonians present most commonly with anorexia, lethargy, wasting, diarrhoea, rhinorrhoea, neurological complications, stomatitis, dermatitis, biliverdinuria, and death (Ariel, 2011; Gibbons & Steffes, 2013; Marschang, 2011). Although no severe or fatal clinical disease were observed in this current study, documentation of novel viruses in wild freshwater turtles is essential to understanding potential threats and providing baseline information on viral epidemiological and clinical characteristics (coinfection variables) for effective management and conservation efforts.

Phylogenetic analysis revealed that the newly discovered HVs (ChelHV1-3) were closely related to chelonid herpesvirus 5, the causative agents of cutaneous fibropapillomatosis. This finding further increased my suspicion that the ChelHVs could be associated with the cutaneous lesions. To evaluate the association of cutaneous lesions observed in freshwater turtles and the presence of HVs, a set of consensus primers and probe were designed to amplify a 124 bp region of the DNA polymerase (DPOL) gene of ChelHVs. A total of 128 freshwater turtles from Alligator Creek and Ross River were tested and 38 were found to be positive (30% prevalence). This study did not find a statistically significant association between the presence of cutaneous lesions and HV real-time PCR status. Given that HVs have been associated with cutaneous lesions in turtles and other species and have gone undetected in some typical HV related cases including fibropapillomatosis (Cowan et al., 2015; Lackovich et al., 1999; Monezi et al., 2016; Monteiro et al., 2021), further investigation to determine the role of ChelHVs (if any) in the pathogenesis of these cutaneous lesions is therefore warranted. The findings in this study suggest that the ChelHVs may be host adapted pathogens associated with subclinical infections; however, cross infections of closely related species can result in serious diseases or enhance the impact of other infections. Future studies should focus on understanding the pathogenic potential of these new viruses as well as their potential impacts on freshwater turtles in Australia. The phylogenetic analysis showed that the novel AdVs clustered with members of the genus *Testadenovirus*. To the best of my knowledge, no clinical disease has been previously associated with any member of this genus. Thus, the novel AdVs were tentatively ruled out as a possible cause of the cutaneous lesions seen in turtles and further analysis was not required.

In Chapter 5, consensus PCR and next generation sequencing (NGS) were complementarily used to discover novel viruses in free-ranging agile wallabies. In this study, one known macropod herpesvirus (MaHV-3) and 14 novel viruses assigned to the families *Adenoviridae*,

Parvoviridae, *Polyomaviridae*, *Picobirnaviridae* and *Circoviridae* were identified. The MaHV-3 had been previously described in eastern grey kangaroos showing signs of respiratory and ocular diseases (Wilcox et al., 2011). Macropod herpesviruses are arguably the most significant pathogens of marsupials, and disease outbreaks have been associated with high mortality events (Callinan & Kefford, 1981; Dickson et al., 1980; Finnie et al., 1976; Wilks et al., 1981). Thus, disease surveillance should be intensified in this population of agile wallabies to ensure a timely formulation of adequate control measures. Although the pathogenic potential of the novel marsupial viruses remains unclear, the genomic characterisation of some of these viruses in this study could help future research on diagnostics, therapeutics, and vaccinology, and improve disease response time in the event of disease outbreaks.

Emerging diseases of zoonotic and conservation importance are often caused by novel viruses, most of which originate from wildlife (Epstein & Anthony, 2017; Murray et al., 2015). This study has shown that NGS (metagenomics) and consensus PCR are important tools for characterising viral diversity in Australian wildlife species. However, the identification of viral sequences and the demonstration of phylogenetic relatedness are not sufficient to predict the pathogenic or zoonotic threats of novel viruses (Epstein & Anthony, 2017). Downstream characterisations such as cell culture and animal disease models are often required to determine the pathogenicity, and zoonotic potential of newly discovered viruses (Epstein & Anthony, 2017; Hu et al., 2021; Park et al., 2020). Establishment of causal relationship between new viruses and diseased populations relies on demonstrating evidence of viral isolates, unique pathological lesions, antigens or nucleic acids in individuals showing clinical signs and/or the fulfillment of Koch's postulate, which includes the isolation of pathogen from sick individuals, propagation in cell culture and disease reproduction in healthy naïve individuals (Epstein & Anthony, 2017; Eyzaguirre etal., 2006; Fredricks &

Relman, 1996; Gardner et al., 1980; Hosainzadegan et al., 2020; Lipkin, 2013; Pfankuche et al., 2018; Walker et al., 2006). Although, in many instances Koch's postulates cannot be fulfilled such as when viruses are unculturable or specific tissue culture is unavailable (Hosainzadegan et al., 2020; Walker et al., 2006). In such instances, the Hill's criteria for causality can be carefully considered (Hill, 1965; Rothman & Greenland, 2005). Hill suggested that causal relationship can be inferred if certain aspects of association are considered and these include, (1) Strength of association: the stronger the association, the more likely that there is a causal relationship; weaker associations does not rule out causal effect. (2) Consistency: if different studies obtain similar results, it become more likely that there is causal relationship. (3) Specificity: specific exposure produces a specific effect. (4) Temporality: the causal exposure should occur before the effect (disease) in time. (5) Biologic gradient: greater exposure level leading to greater incidence is most likely associated but not necessarily the cause. (6) Plausibility: plausible mechanism between cause and effect useful but limited by current knowledge. (7) Coherence: presence of other biological effects that are relevant to the causal pathway, increases the likelihood of causal relationship. (8) Experimental evidence: refers to reduction in the disease rate following removal of the causative agent. (9) Analogy: if similar agents produce similar effects, then it is more likely that there is a causal relationship (Asokan & Asokan, 2016; Hill, 1965). More detailed explanation of these criteria has been provided by Rothman and Greenland (2005). Unfortunately, these nine criteria are saddled with uncertainty and reservations in reaching definitive conclusions on causation (Rothman & Greenland, 2005).

For these reasons, I have proposed a workflow for the effective discovery and reporting of novel viruses in both diseased and healthy wildlife species (Figure 6.1). In brief, following sample collection and depending on the health status of wildlife species as well as the study objectives, samples should be processed for NGS or consensus PCR detection of novel

viruses. To ensure that false positive results are not reported, PCR assays should be used to confirm the presence of novel viruses identified by NGS. When dealing with difficult to diagnose or unknown cases in diseased species, NGS should be done if the differential diagnosis is broad; otherwise, consensus PCR can be used to identify the potential causative viruses within a given taxa (narrow differential diagnosis). Establishment of causal relationship can be directly determined through laboratory characterisation of novel viruses using cell culture or animal models. Viruses can also be detected and localised in diseased tissues (as evidence of causation) using immuno-histochemical, immunofluorescent (viral antigen) or in situ hybridisation (nucleic acids) techniques prior or in complementary to viral propagation in tissue cultures or animals. Where these laboratory approaches are unattainable, indirect evidence of causation can be obtained through statistical inference or mathematic modelling of the disease. The statistical inference of causation was exemplified in Chapter four where I used odds ratio to determine the possible association between novel HVs and the presence of cutaneous lesions in freshwater turtles. Although, statistical analysis showed no association, I could not rule out HV as a possible causative agent. This was in accordance with Hill's viewpoint, which suggested that absence of association should not rule out the causal relationship (Asokan & Asokan, 2016). Only when novel viruses have been confirmed to be associated with a disease (pathogenic or zoonotic), can epidemiological characterisation be conducted to determine the risk factors associated with potential disease outbreaks. Results from inferential inductions, and laboratory analyses as well as epidemiological characterisations of important novel viruses should be directly or indirectly (through peer reviewed publications) disseminated to government agencies or decision makers to inform surveillance and outbreak response plans (Figure 6.1). Viral discovery studies in healthy wildlife species are often conducted to monitor disease emergence. The approach used for viral discovery in healthy wildlife is similar to that of diseased species

except that there is no requirement to associate causation. However, important novel virus from healthy wildlife species should be characterised to provide insight into their pathogenic, zoonotic, or epidemiologic potential. Where this is not possible, novel viruses should still be reported as they can serve as potential candidates for vaccines and diagnostics in events of disease outbreak (Chapter 5).

The overarching aim of this thesis was to evaluate viral discovery approaches, obtain evidence of circulating novel viruses, and propose a suitable workflow for identifying, characterising, and reporting novel viruses in diseased and healthy wildlife populations. This project developed sensitive consensus PCR assays and deployed these tools in combination with other universal detection assays to identify and characterise a total of 20 novel viruses in freshwater turtles and agile wallabies. The suggested workflow (Figure 6.1) for viral discovery in diseased and healthy wildlife species can be a valuable surveillance protocol for future investigations. The findings and recommendations of this research have significant implications for the design and implementation of wildlife conservation and disease monitoring programmes.

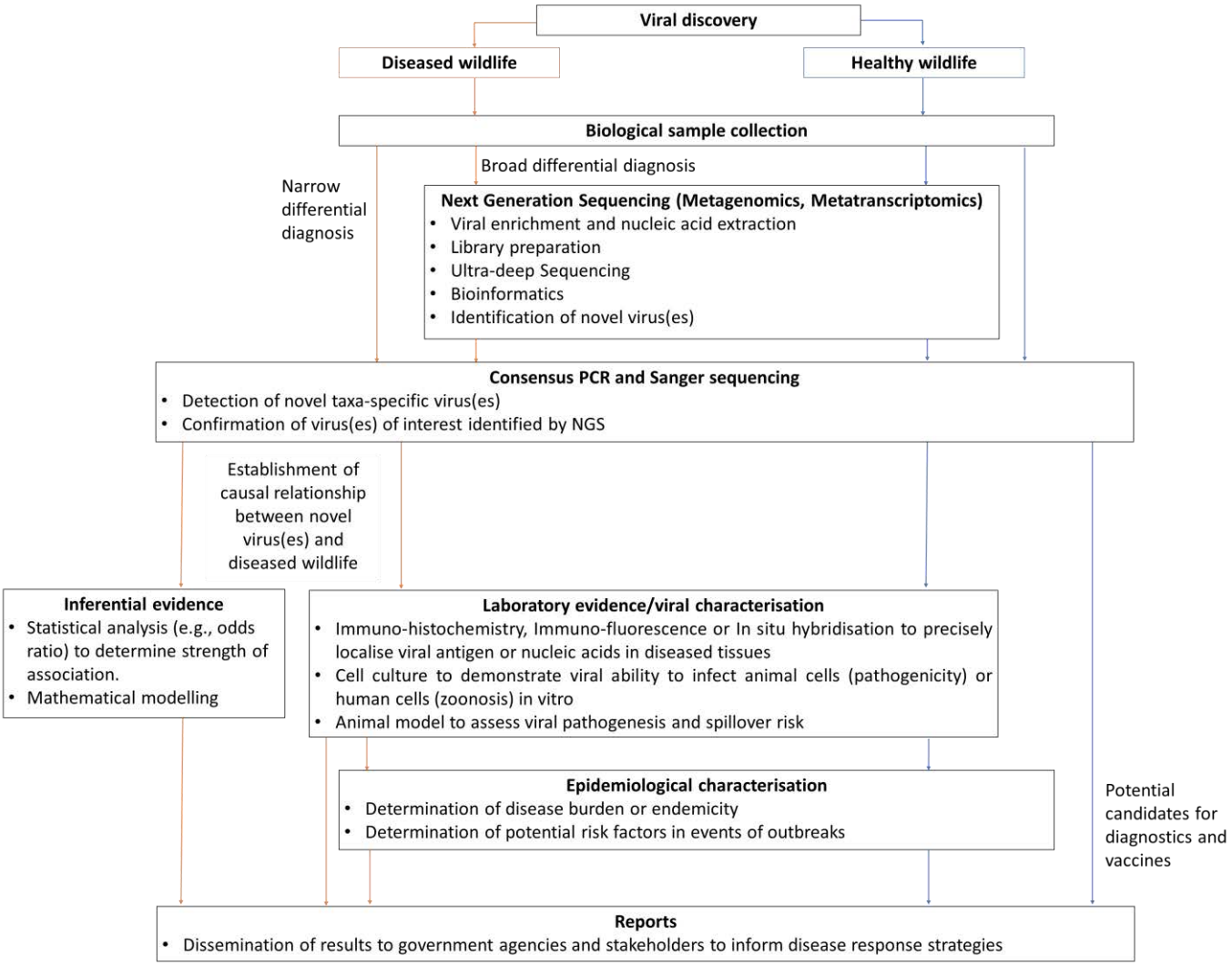


Figure 6. 1. A workflow chart illustrating the use of pathogen discovery tools to investigate the occurrence of new viruses in diseased and healthy wildlife.

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Appendices: List of Supplementary Figures

Appendices: List of Supplementary Tables

Appendix One: Supplementary Files from Chapter Two

Supplementary Table 2. 1. Literature review search terms and strategies.

A. Terms searched on Medline and Scopus

1. Fibropapillomatosis OR grey-patch disease OR herpesviridae infections OR herpesvirus infection OR loggerhead genital-respiratory herpesvirus OR herpesvirus disease AND Reptiles
2. Fibropapillomatosis OR grey-patch disease OR herpesviridae infections OR herpesvirus infection OR loggerhead genital-respiratory herpesvirus OR herpesvirus disease AND Turtles

3. Fibropapillomatosis OR grey-patch disease OR herpesviridae infections OR herpesvirus infection OR loggerhead genital-respiratory herpesvirus OR herpesvirus disease AND Tortoise
4. Fibropapillomatosis OR grey-patch disease OR herpesviridae infections OR herpesvirus infection OR loggerhead genital-respiratory herpesvirus OR herpesvirus disease AND Snakes
5. Fibropapillomatosis OR grey-patch disease OR herpesviridae infections OR herpesvirus infection OR loggerhead genital-respiratory herpesvirus OR herpesvirus disease AND Alligators and Crocodiles
6. Fibropapillomatosis OR grey-patch disease OR herpesviridae infections OR herpesvirus infection OR loggerhead genital-respiratory herpesvirus OR herpesvirus disease AND Lizards

B. Terms searched on PubMed and Scopus

1. Herpesvirus reptiles
2. Herpesvirus turtles
3. Herpesvirus tortoise
4. Herpesvirus Snakes
5. Herpesvirus alligators and crocodiles
6. Herpesvirus lizards
7. Fibropapillomatosis reptiles
8. Grey patch disease reptiles
9. Loggerhead genital-respiratory herpesvirus reptiles

Supplementary Table 2. 2. Data extracted for literature review.

First Author	Date	HV spp.	Reptile species	Diagnostic Method
Clark	1972	IgHV-1	*Iguana iguana*	Virus isolation and Virus neutralization
Zeigel	1972	IgHV-1	*Iguana iguana*	Virus isolation and Electron Microscopy
Rebell	1975	ChHV-1	*Chelonia mydas*	Virus isolation, Histopathology and Electron Microscopy
Raynaud	1976	Not characterised	*Lacerta viridis*	Electron Microscopy
Frye	1977	ChHV-2	*Clemmys marmorata*	Histopathology and Electron Microscopy
Haines	1977	ChHV-1	*Chelonia mydas*	Electron Microscopy
Simpson	1979	Elapid HV-1	*Naja naja kaouthia*	Histopathology and Electron Microscopy
Cox	1980	ChHV-3	*Chrysemys picta*	Histopathology and Electron Microscopy
Harper	1982	Not characterised	*Gopherus agassizii*	Histopathology and Electron Microscopy
Jacobson	1982	ChHV-3?	*Glyptemys barbouri* and *Glyptemys pseudogeographica*	Histopathology and Electron Microscopy
Hauser	1983	Not characterised	Boa constrictor	Histopathology and Electron Microscopy
Jacobson	1985	ChHV-4	*Geochelone chilensis*	Histopathology and Electron Microscopy
Jacobson	1986	ChHV-6	*Chelonia mydas*	Virus isolation, Histopathology and Electron Microscopy
Braune	1989	Not characterised	*Tortoises*	Histopathology and Electron Microscopy
Lange	1989	Not characterised	*Testudo hermanni* and *Agrionemys horsfieldii*	Virus isolation and Electron Microscopy
Müller	1990	Not characterised	*Spur-tailed and Spur-thighed*	Histopathology
Watson	1993	Not characterised	*Agama agama*	Histopathology and Electron Microscopy
Pettan-Brewer	1996	Not characterised	*Gopherus agassizii*	Histopathology and Electron Microscopy
Marschang	1997	TeHV-3	*Testudo hermanni and T. graeca*	Virus isolation, Histopathology and Virus Neutralization tests

Drury	1998	Not characterised	*Testudo hermanni and Geochelone pardalis*	Virus isolation, Histopathology and Electron Microscopy
Herbst	1998	ChHV-5	*Chelonia mydas*	Immunohistochemical assay.
Muro	1998	Not characterised	*Testudo graeca graeca*	Histopathology and Electron Microscopy
Quackenbush	1998	ChHV-5	*Chelonia myda, Caretta caretta* and *Lepidochelys olivacea*	Nested consensus PCR, species-specific PCR and Southern blot analysis
Aguirre	1999	ChHV-5	*Lepidochelys olivacea*	Histopathology
Drury	1999	Not characterised	*Testudo graeca*	Virus isolation and Electron Microscopy
Herbst	1999	ChHV-5	*Chelonia mydas*	Immunohistochemical assay and Histopathology
Kübber-Heiss	1999	Not characterised	*Testudo hermanni, Agrionemys horsfieldii* and *Testudo graeca*	Virus isolation, Histopathology and Electron Microscopy
Lackovich	1999	ChHV-5	*Chelonia myda* and *Caretta caretta*	Virus Isolation, Histopathology and Nested consensus PCR
Nie	1999	Not characterised	Trionyx sinensis (Chinese soft-shelled turtles)	Virus neutralization test and Dot-ELISA
Une	1999	Not characterised	Malacochersus tornieri (pancake tortoises) and Testudo horsfieldii	Histopathology, Electron Microscopy & Nested consensus PCR
Lu	2000	ChHV-5	*Chelonia mydas*	Nested species-specific PCR
Teifke	2000	Not characterised	*T. horsfieldii* and *T. hermanni*	Virus isolation, Histopathology, Electron Microscopy, specific PCR and nonradioactive in situ hybridization
Une	2000	TeHV-1	*Malacochersus tornieri, Testudo horsfieldii and Testudo graeca,Geochelone pardali* and *Geoche-lone carbonaria*	Histolopathology and Nested and heminested consensus PCR

Coberley	2001	ChHV-6	*Chelonia mydas*	ELISA, Western immunoblotting and Immunohistochemistry
Coberley	2001	ChHV-6	*Chelonia mydas*	ELISA and Immunoblotting
Marschang	2001	TeHV-3	*Testudo graeca, T. horsfieldii, T. hermanni, T. marginata*	Virus neutralization tests and Restriction endonuclease analysis
Marschang	2001	Not characterised	*Testudo graeca, T. hermanni, T. marginata*	Virus isolation and virus neutralization test
Murakami	2001	TeHV-1	M. tornieri	Nested consensus PCR and species-specific PCR (non-nested and heminested)
Origgi	2001	Not characterised	*Testudo graeca, T. hermanni*	ELISA, Virus neutralization tests and Western immunoblotting
Quackenbush	2001	ChHV-5	*Chelonia myda, Lepidochelys olivacea* and *Caretta caretta*	Species-specific PCR and Quantitative real time PCR (qPCR)
Yu	2001	ChHV-5	*Chelonia mydas*	genomic walking method based on restriction enzyme digestion, self-ligation and inverse polymerase chain reaction
Hervas	2002	Not characterised	T. horsfieldii	Histopathology and Electron Microscopy
Lu	2003	ChHV-5	*Chelonia mydas*	Species-specific nested PCR, Reverse transcriptase-PCR (RT-PCR) and quantitative RT-PCR
Origgi	2003	TeHV-3	*Testudo graeca, T. hermanni*	Indirect and direct immunoperoxidase assays
Wellehan	2003	IgHV-2	*Sauromalus varius* (lizards)	Histopathology, Electron Microscopy and Nested consensus PCR
Greenblatt	2004	ChHV-5	*Chelonia mydas*	qPCR, qRT-PCR
McCowan	2004	Not characterised	*Crocodylus porosus*	Histopathology, Electron Microscopy
Nigro	2004	ChHV-5	*Chelonia mydas*	Species-specific nested PCR, genomic walking method based on restriction enzyme

				digestion, self-ligation and inverse polymerase chain reaction
Nigro	2004	ChHV-5	*Chelonia mydas*	genomic walking method based on restriction enzyme digestion, self-ligation and inverse polymerase chain reaction
Origgi	2004	TeHV-3	*Testudo graeca*	Virus isolation, Histopathology, Electron Microscopy, ELISA, Virus neutralization tests, Immunohistochemistry and species-specific, Semiquantitative PCR, and RT-PCR
Work	2004	ChHV-5	*Chelonia mydas*	Histopathology
Soares	2004	Not characterised	Testudo spp.	Nonradioactive In situ hybridization
Wellehan	2004	Gerrhosaurid herpesvirus 1, 2, 3	*Gerrhosaurus major, G. nigrolineatus*	Histopathology and Nested consensus PCR
Ene	2005	ChHV-5	*Chelonia mydas*	Species-specific PCR
Greenblatt	2005	ChHV-5	*Chelonia mydas*	Species-specific PCR
Johnson	2005	TeHV-2	*Gopherus agassizii*	Histopathology, Electron Microscopy, ELISA and Nested consensus PCR and species-specific PCR
Wilkinson	2005	IgHV-1	*Iguana iguana*	Histopathology and Electron Microscopy
Wellehan	2005	VHV-1	*Varanus prasinus*	Nested consensus PCR and DNA in situ hybridization
Hunt	2006	Not characterised	*Testudo graeca, T. hermanni, T. kleinmanni,T, marginata, and T. horsfieldi*	Virus isolation, Histopathology, and PCR
Johnson	2006	TeHV-1?	*Gopherus agassizii*	ELISA
Marschang	2006	TeHV-3	*T. graeca, T. hermanni, T. horsfieldii*	Nested consensus PCR, species-specific PCR and DNA in situ hybridization
Fischer	2006	Not characterised	Agrionemys horsfieldii	Histopathology and PCR
Benetka	2007	Not characterised	*Geochelone pardalis pardalis*	Species-specific PCR

Marschang	2007	TeHV-3?	*Testudo graeca*	Virus neutralization test
Herbst	2008	ChHV-5	*Chelonia mydas, Caretta caretta*	ELISA
Kang	2008	ChHV-5	Chelonia mydas	Histopathology and in-situ hybridization
Stacy	2008	LGRV and LOCV	*Caretta caretta*	Histopathology and Nested consensus PCR
Marschang	2009	Not characterised	*Testudo kleinmanni, Testudo graeca*	Virus isolation, species-specific and Nested consensus PCR
Martel	2009	Not characterised	Tortoise spp.	Virus neutralization test and Nested consensus PCR
Work	2009	ChHV-5	*Chelonia mydas*	Virus isolation and qPCR
Bicknese	2010	TeHV-4	*Chersina angulata*	Nested consensus PCR
Hughes-Hanks	2010	VHV-3	*Varanus spp.*	Histopathology, Electron Microscopy and Nested consensus PCR
Literak	2010	*Lacerta viridis HV-1*	*Lacerta viridis*	Histopathology, Electron Microscopy and Nested consensus PCR
Stöhr	2010	TeHV-1	*Testudo hermanni boettgeri*	Virus solation, Virus neutralization test, Nested consensus PCR and species-specific PCR
Salinas	2011	ChHV type I and type II	*Testudo graeca, Testudo ibera, Agryonemys horsfieldii*	Nested consensus PCR and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)
Ackermann	2012	ChHV-5	Chelonia mydas)	Conventional species-specific PCR, qPCR and RT-PCR
Duarte	2012	ChHV-5	Chelonia mydas)	Histopathology and Conventional seminested PCR
Jacobson	2012	TeHV2 and TeHV3	*Gopherus agassizii*	Histopathology, Indirect ELISA, Nested consensus PCR and species-specific PCR
Page-Karjian	2012	ChHV-5	*Chelonia mydas*	Species-specific primary and nested PCRs
Patricio	2012	ChHV-5	*Chelonia mydas*	Species-specific PCR
Rodenbusch	2012	ChHV-5	*Chelonia mydas*, *Caretta caretta*	Histology, Species-specific PCR and Quantitative real time PCR (qPCR)

Silbernagel	2013	Not characterised	Emys marmorata and Trachemys scripta elegans	Nested consensus PCR
Alfaro-Núñez	2014	ChHV-5	*Chelonia mydas*	Nested consensus PCR, species-specific singleplex and nested PCRs
Alfaro-Núñez	2014	ChHV-5	*Chelonia myda, Lepidochelys olivacea* and *Caretta caretta, Eretmochelys imbricata, Dermochelys coriacea*	Species-specific singleplex PCR
Braun	2014	TeHV-2	*Gopherus agassizii*	Real-time qPCR
Catoi	2014	Not characterised	*Vipera ammodytes ammodytes*	Histopathology and Electron Microscopy
Jungwirth	2014	EmyHV-1	*Pseudemys concinna concinna*	Histopathology, Electron Microscopy and Nested consensus PCR
Page-Karjian	2014	ChHV-5	*Chelonia mydas*	Histopathology and PCR
Rodenbusch	2014	ChHV-5	*Chelonia mydas*	Histopathology and Species-specific PCR and real time qPCR
Cowan	2015	Not characterised	Emydura macquarii krefftii	Histopathology and Nested consensus PCR
Gandar	2015	TeHV-3	*Testudo horsfieldii, T. hermanni*	Virus isolation, Electron microscopy and Species-specific PCR and real time qPCR
Hyndman	2015	CrHV-1, CrHV-2, CrHV3	*Crocodylus porosus* and *Crocodylus johnstoni*	virus isolation and Nested consensus PCR and species-specific single round and duplex PCR
Marschang	2015	Pelomedusid HV-1	*Pelusios castaneus*	PCR
Origgi	2015	TeHV-3	*Testudo horsfieldii, T. hermanni, S. pardalis, T. graeca and testudo sp.*	Virus solation and Nested consensus PCR and species-specific PCR
Ossiboff	2015	EmyHV-1	*Graptemys geographica, Trachemys scripta elegans, Clemmys guttata, Chrysemys picta*	Histopathology and Nested consensus PCR

Ossiboff	2015	GlyHV-1, GlyHV-2, EmyHV-2	*Glyptemys muhlenbergii, G. insculpta, Clemmys guttata*	Nested consensus PCR
Page-Karjian	2015	ChHV-5	*Chelonia mydas*	qPCR
Sim	2015	TerHV-1	Terrapene carolina carolina	Histopathology and Nested consensus PCR
Work	2015	ChHV-5	*Chelonia mydas*	Histopathology and Immunohistochemistry, Western blot and species-specific PCR
Yonkers	2015	TerHV-2	*Terrapene carolina*	Histopathology and Nested consensus PCR
Alfaro-Núñez	2016	ChHV-5	*Chelonia mydas*	Real-time qPCR
Goe	2016	HeHV-1	*Heloderma suspectum cinctum*	Histopathology and Nested consensus PCR
Kane	2016	TerHV-1	*Terrapene carolina carolina*	Nested consensus PCR, Real time qPCR
Kolesnik	2016	TeHV-4	*Stigmochelys pardalis*	PCR
Monezi	2016	ChHV-5	*Chelonia mydas*	Histopathology and Nested species-specific PCR
Shilton	2016	CrHV-1, CrHV-2	*Crocodylus porosus*	Virus isolation, Histopathology, electron microscopy and Nested consensus PCR
Archer	2017	TerHV-1	*Terrapene carolina carolina*	Multiplex qPCR
Ariel	2017	ChHV-5	*Chelonia mydas, Caretta caretta*	Histopathology and Species-specific PCR
Chaves	2017	ChHV-5	*Chelonia myda, Lepidochelys olivacea*	Nested species-specific PCR
Kane	2017	TerHV-1	*Terrapene carolina carolina*	qPCR
Kolesnik	2017	TeHV-1, TeHV-3, TeHV-4	Tortoise spp. And freshwater turtle spp.	Nested consensus PCR, Species-specific PCR and DNA in situ hybridization
Li	2017	ChHV-5	*Chelonia mydas*	Histopathology and species-specific PCR, RT-PCR
Page-Karjian	2017	ChHV-5	*Chelonia mydas*	qPCR
Work	2017	ChHV-5	*Chelonia mydas*	Histopathology, Electron microscopy and Immunohistochemistry
Adamovicz	2018	TerHV-1	*Terrapene carolina carolina*	Virus solation and qPCR

Lawrance	2018	ChHV-5	*Chelonia mydas, Caretta caretta*	qPCR, species specific nested PCR
Lindemann	2018	EBHV-1	*Emydoidea blandingii*	Nested consensus PCR and qPCR
Marenzoni	2018	TeHV-3	*T. hermanni, T. graeca, T. horsfieldii, T. marginata, Trachemys spp., Emys orbicularys, Graptemys spp., Pseudemys spp., Cuora amboinensis and Terrapene carolina mayor*	Histopathology, ELISA, Nested consensus PCR, Species-specific PCR and semi-nested PCR
Morrison	2018	ChHV-5	*Chelonia mydas*	Long-range PCR
Willimann	2018	ChHV-5	*Chelonia mydas*	ELISA
Siroky	2018	Not characterised	*Pelusios williamsi*	Histopathology, Electron Microscopy and Nested consensus PCR
Aplasca	2019	Chelydra herpesvirus 1, TerHV-1?	*Chelydra serpentina* and *Trachemys scripta*	Nested consensus PCR
Cardenas	2019	ChHV-5	*Chelonia mydas, Lepidochelys olivacea*	nested PCR
Diaz-Delgado	2019	ChHV-5	*Dermochelys coriacea*	Species-specific PCR
Domiciano	2019	ChHV-5	*Chelonia mydas*	Histopathology and Species-specific PCR
Lindemann	2019	EBHV1	*Emydoidea blandingii*	qPCR
Lovstad	2019	Opheodrys herpesvirus 1	Opheodrys vernalis	Histopathology, Nested consensus PCR and in-situ hybridization
Mejia-Radillo	2019	ChHV-5	Lepidochelys olivacea	Species-specific PCR
Jones	2020	ChHV-5	*Chelonia mydas, Caretta caretta, Eretmochelys imbricata*	Species-specific PCR
Work	2020	ChHV-5	*Chelonia mydas*	ELISA

Supplementary Table 2. 3. Data extracted for literature review.

Author	year	Sample	Test	Biomarker	Virus	Classification	Host (common name)	Number tested	Number infected	Positive/number showing clinical signs	Confirmed mortality	Observed clinical signs	Status	Location
Joyce	2022	Blood and tissues	NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	33	33		0	None	captive	Queensland
Joyce	2022	Blood and tissues	NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	33	33		0	None	captive	New South Wales
Bowater	2022	Tissues	Gross-, histo-pathology, pan-PCR	Virus (IIB, nucleic acid)	Phascolarctid alphaherpesvirus 3	Herpesviridae	Koala	1	1	1+/1	0	Lethargy, weakness, anorexia and severe dyspnoea	captive	Queensland

Blyton, Pyne	2022	Blood	qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	151	151		0	Urinary leakage/cystitis, renal pathology, reproductive pathology and urogenital pathology	wild	Queensland
Blyton, Young	2022	Feces	qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	59	59		0	None	wild	Queensland
Blyton	2022	Feces	qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	39	39		0	None	wild	New South Wales
Tarlinton	2022	Tissues (spleen)	Nanopore	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	3	3		0	None	wild	Victoria
Tarlinton	2022	Tissues (liver)	Nanopore	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	1	1		0	None	captive	South Australia
Tarlinton	2022	Tissues (lymph node)	Long- range PCR and nanopore	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	1	1		0	None	wild	South Australia
Tarlinton	2022	Tissues (Submandibular lymph node), blood	NGS (RNAseq) and PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	10	10		0	None	wild	Queensland
Tarlinton	2022	Tissues (Submandibular lymph), blood	NGS (RNAseq) and PCR	Virus (nucleic aicid)	Koala retrovirus	Retroviridae	Koala	19	18		0	None	wild	South Australia

Blyton	2022	feces, blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	39	39		0	None	wild	New South Wales
Joyce	2021	Blood	NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	109	109		0	None	captive	Queensland
McEwen	2021	Tissues	Inverse PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	10	10	10+/10	0	Neoplasia (Lymphoma/leukaemia, tumour in viscera organs, Mammary tumour, Rhabdomyoma, Fibrosarcoma, Anaplastic carcinoma, Osteochondroma).	captive	Queensland
Johnson	2021	Sera	virus neutraliation	Antibody	Ross river virus	Togaviridae	Koala	218	183		0	None	wild	Queensland
Quigley	2021	blood, urogenital swab, feces	PCR, NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	38	38		0	None	wild	Queensland
Quigley	2021	Feces	PCR, NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	37	37		0	None	wild	New South Wales
Quigley	2021	Feces	PCR, NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	274	42		0	None	wild	Victoria

Martínez-Pérez	2021	Blood	virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Quokka	142	1		0	None	wild	Western Australia
Martínez-Pérez	2021	Blood	virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Quokka	142	1		0	None	wild	Western Australia
Martínez-Pérez	2021	Blood	PCR	Virus (nucleic acid)	Macropod herpesvirus 6	Herpesviridae	Quokka	121	13		0	None	wild	Western Australia
Langhorne	2021	blood, oropharyngeal swabs, and urine	PCR	Virus (nucleic acid)	Peramelid herpesvirus 2	Herpesviridae	Bandicoot	35	18		0	None	wild	Queensland
Zheng	2020	blood, tissues	qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	78	78		0	None	captive	Australia (unknown state)
Zheng	2020	Blood	qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	27	27		0	None	captive	Queensland
Zheng	2020	Blood	qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	21	21		0	None	wild	Queensland
Tolpinrud	2020	Sera	Indirect ELISA	Antibody	Wobbly possum disease virus	Arteriviridae	Possum	188	30		0	None	wild	Victoria
Tolpinrud	2020	tissue (spleen)	qPCR	Virus (nucleic acid)	Wobbly possum disease virus	Arteriviridae	Possum	199	0		0	None	wild	Victoria
Sarker, Fabijan	2020	Blood	conventional PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	71	71	9+/9	0	neoplasia	captive	Queensland
Sarker, Fabijan	2020	Blood	conventional PCR and qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	97	96	5+/5	0	Neoplasia	captive	South Australia
Robbins	2020	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	80	80		0	None	wild	Queensland
Porter	2020	Tissue	NGS	Virus (nucleic acid)	Koala hepaci-like virus	Flaviviridae	Koala	1	1		0	Severe chlamydiosis	wild	New South Wales
Martínez-Pérez	2020	Blood	PCR	Virus (nucleic acid)	Macropod herpesvirus 6	Herpesviridae	Quokka	58	13		0	None	wild	Western Australia
Kasimov	2020	oropharyngeal swabs, spleen	PCR	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	167	106		0	None	wild	South Australia

Kasimov	2020	oropharyngeal swabs, spleen	PCR	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 2	Herpesviridae	Koala	167	40		0	None	wild	South Australia
Shima	2020	Serum	virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Kangaroo	35	0		0	None	wild	Queensland
Shima	2020	Serum	virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Lumholtz's Tree-kangaroo	35	0		0	None	wild	Queensland
Shima	2020	Serum	virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Kangaroo	9	4		0	None	captive	Queensland
Shima	2020	Serum	virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Lumholtz's Tree-kangaroo	9	4		0	None	captive	Queensland
Shima	2020	tissue swabs	PCR, histopathology	Virus (nucleic acid)	Lumholtz's Tree-kangaroo herpesvirus	Herpesviridae	Kangaroo	12	11		0	None	wild	Queensland
Shima	2020	tissue swabs	PCR, histopathology	Virus (nucleic acid)	Lumholtz's Tree-kangaroo herpesvirus	Herpesviridae	Kangaroo	8	6	1+/1	0	lethargy, tachypnea, dyspnea in one positive individual	captive	Queensland
Shima	2020	tissue swabs	PCR, histopathology	Virus (nucleic acid)	Macropod herpesvirus 4	Herpesviridae	Kangaroo	20	1	1+/1	1	Acute respiratory signs, oculo-nasal discharge and death	captive	Queensland
Sarker	2020	Blood	cell culture and qPCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	15	10	1+/1	0	Leukaemia	captive	Queensland
Fabijan	2020	tissues, blood	Histopathology, Immunohistochemistry, PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	46	46	9+/9	0	Neoplasia (lymphoma and lymphoid leukaemia, mesothelioma, osteochondroma)	wild	Queensland
Fabijan	2020	tissues, blood	Histopathology, Immunohistochemistry, PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	21	21		0	None	captive	Queensland

Fabijan	2020	tissues, blood	Histopathology, Immunohistochemistry, PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	93	92	5+/5	0	Neoplasia (lymphoma, osteochondroma)	wild	South Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	Ross river virus	Togaviridae	Western grey kangaroo	100	59		0	None	wild	Western Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	Barmah Forest virus	Togaviridae	Western grey kangaroo	100	48		0	None	wild	Western Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	Sindbis virus	Togaviridae	Western grey kangaroo	100	0		0	None	wild	Western Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	Alfuy virus	Flaviviridae	Western grey kangaroo	100	10		0	None	wild	Western Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	Stratford virus	Flaviviridae	western grey kangaroo	100	8		0	None	wild	Western Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	Edge Hill virus	Flaviviridae	Western grey kangaroo	100	6		0	None	wild	Western Australia
Gyawali	2020	sera	plaque reduction neutralization test	Antibody	Murray valley encephalitis virus	Flaviviridae	Western grey kangaroo	100	1		0	None	wild	Western Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	Kokobera virus	Flaviviridae	Western grey kangaroo	100	1		0	None	wild	Western Australia
Gyawali	2020	Sera	plaque reduction neutralization test	Antibody	West Nile virus (kunjin strain)	Flaviviridae	Western grey kangaroo	100	0		0	None	wild	Western Australia
Olagoke	2019	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	235	235		0	None	wild	Queensland
Olagoke	2019	Blood	ELISA	Antibody	Koala retrovirus	Retroviridae	Koala	235	223		0	None	wild	Queensland
Harvey	2019	RNAseq libraries	NGS	Virus (nucleic acid)	Burpengary Virus	Picornaviridae	Koala	26	15	0	0	Ocular disease	captive	Queensland
Chang	2019	tissue (brain, liver and kidney)	PCR, NGS	Virus (nucleic acid)	wobbly possum disease virus	Arteriviridae	Possum	9	3	3+/9	0	Nystagmus, blindness,	wild	New South Wales

												abnormal gait, alopeicia		
Chang	2019	tissue (brain, liver and kidney)	PCR, NGS	Virus (nucleic acid)	Hepacivirus	Flaviviridae	Brushtail possum	9	5		0	nystagmus, blindness, abnormal gait, alopeicia	wild	New South Wales
Vaz	2019	swabs (nasal, oropharyngeal, and ocular, cloacal and urogenital)	PCR (HRM)	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	773	130		0	Non-HV signs (urinary tract abnormality, reproductive abnormality, Wet Bottom)	wild	Victoria
Vaz, Legione	2019	swabs (nasal, oropharyngeal, and ocular, cloacal and urogenital)	PCR (HRM)	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	11	5		0	Non-HV signs (urinary tract abnormality, reproductive abnormality, Wet Bottom)	captive	Victoria
Vaz	2019	swabs (nasal, oropharyngeal, and ocular, cloacal and urogenital)	PCR (HRM)	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	26	6		0	Non-HV signs (urinary tract abnormality, reproductive abnormality, Wet Bottom)	unknown	Victoria
Vaz	2019	swabs (nasal, oropharyngeal, and ocular, cloacal and urogenital)	PCR (HRM)	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 2	Herpesviridae	Koala	773	179		0	Non-HV signs (urinary tract abnormality, reproductive abnormality, Wet Bottom)	wild	Victoria
Vaz, legione	2019	Swabs (nasal, oropharyngeal, and ocular, cloacal and urogenital)	PCR (HRM)	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 2	Herpesviridae	Koala	11	0		0	Non-HV signs (urinary tract abnormality, reproductive abnormality, Wet Bottom)	captive	Victoria
Vaz	2019	Swabs (nasal, oropharyngeal, and ocular, cloacal and urogenital)	PCR (HRM)	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	26	4		0	Non-HV signs (urinary tract abnormality, reproductive abnormality, Wet Bottom)	unknown	Victoria

Vaz	2019	Oropharyngeal swabs	virus isolation and NGS	Virus (CPE and nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	1	1		0	None	unknown	Victoria
Vaz	2019	Nasal swabs	Virus isolation and NGS	Virus (CPE and nucleic acid)	Vombatid gammaherpesvirus 1	Herpesviridae	Wombat	1	1		0	None	unknown	Victoria
Sarker	2019	Blood	PCR and NGS	Virus (nucleic aicid)	Koala retrovirus	Retroviridae	Koala	33	33	8+/8	0	Neoplasia	captive	Queensland
Sarker	2019	Blood	PCR and NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	28	28	5+/5	0	Neoplasia	captive	South Australia
Quigley	2019	Blood, urogenital swabs, and ocular swabs	PCR and NGS	Virus (nucleic aicid)	Koala retrovirus	Retroviridae	Koala	16	16		0	Chlamydial cystitis	wild	Queensland
Fabijan	2019	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	245	121	1+/1	0	Lymphosarcoma	wild	South Australia
Quigley	2018	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	290	290	4+/5	0	Neoplasia (lymphoma, osteochondroma, mesothelioma, lymphoid leukemia, lymphoma)	wild	Queensland
Waugh	2017	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	36	36		0	Chlamydial disease	wild	Queensland
Skogvold	2017	Conjunctival, nasal, oropharyngeal, cloacal,	PCR	Virus (nucleic acid)	Potoroid Herpesvirus 1	Herpesviridae	Woylie	33	3		0	None	wild	Western Australia

		and preputial												
Skogvold	2017	conjunctival, nasal, oropharyngeal, cloacal, and preputial	PCR	Virus (nucleic acid)	Potoroid Herpesvirus 1	Herpesviridae	Woylie	12	0		0	None	captive	Western Australia
Skogvold	2017	Blood	virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Woylie	10	0		0	None	wild	Western Australia
Skogvold	2017	Blood	virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Woylie	58	2		0	None	captive	Western Australia
Skogvold	2017	Blood	virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Woylie	59	1		0	None	wild	Western Australia
Skogvold	2017	Blood	virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Woylie	9	0		0	None	captive	Western Australia
Skogvold	2017	Blood	virus neutralisation	Antibody	Wallal virus	Sedoreoviridae	Woylie	54	0		0	None	wild	Western Australia
Skogvold	2017	Blood	virus neutralisation	Antibody	Wallal virus	Sedoreoviridae	Woylie	12	0		0	None	captive	Western Australia
Sarker	2017	Tissues	NGS and electron microscopy	Virus (nucleic aicid, virus particles)	Eastern grey kangaroopox virus	Poxviridae	Eastern grey kangaroo	1	1	1+/1	0	Skin lesions (nodule)	wild	New South Wales
Legione	2017	blood and tissues	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	648	160		0	Chlamydial disease	wild	Victoria
Hobbs	2017	Tissue	PacBio, PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	1	1		0	Chlamydial disease	wild	New South Wales
Chappell	2017	Blood	NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	18	18		0	None	wild	Queensland
Bennett	2017	Tissues	NGS	Virus (nucleic acid)	Eastern grey kangaroopox virus	Poxviridae	Eastern grey kangaroo	1	1		0	Papillomatous skin	wild	Queensland
Bennett	2017	Tissues	NGS	Virus (nucleic acid)	Western grey kangaroopox virus	Poxviridae	Western grey kangaroo	1	1		0	Skin mass	wild	Western Australia

Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Vombatid gammaherpesvirus 1	Herpesviridae	Common wombat	33	5		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Vombatid herpesvirus 2	Herpesviridae	Common wombat	33	7		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Vombatid herpesvirus 3	Herpesviridae	Common wombat	33	3		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Macropod herpesvirus 5	Herpesviridae	Swamp wallaby	15	4		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce)	PCR	Virus (nucleic acid)	Dasyurid herpesvirus 2	Herpesviridae	Tasmanian devil	21	2		0	None	wild	Tasmania
Stalder	2015	swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce)	PCR	Virus (nucleic acid)	Dasyurid herpesvirus 2	Herpesviridae	Tasmanian devil	29	15		0	None	captive	Tasmania

Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Peramelid herpesvirus 1	Herpesviridae	Southern brown bandicoot	11	1		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Macropod herpesvirus 3	Herpesviridae	Eastern grey kangaroo	96	19		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Macropod herpesvirus 4	Herpesviridae	Eastern grey kangaroo	96	5		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	99	10		0	None	no clear	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 2	Herpesviridae	Koala	99	23		0	None	not clear	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	unknown	Herpesviridae	Brush-tailed rock wallaby	3	0		0	None	wild	Victoria

Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	unknown	Herpesviridae	Brush-tailed rock wallaby	10	0		0	None	captive	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Tammar wallaby	8	0		0	None	captive	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Yellow-footed rock wallaby	3	0		0	None	captive	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Red-necked wallaby	1	0		0	None	captive	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Long-nosed potoroo	9	0		0	None	captive	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Eastern quoll	1	0		0	None	wild	Victoria

Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Eastern quoll	1	0		0	None	captive	Victoria
Stalder	2015	swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	virus (nucleic acid)	Unknown	Herpesviridae	Eastern barred bandicoot	3	0		0	None	captive	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Brushtail possum	2	0		0	None	wild	Victoria
Stalder	2015	Swab (conjunctivae, nasal cavity, oropharynx, cloaca, prepuce), blood	PCR	Virus (nucleic acid)	Unknown	Herpesviridae	Common ringtail possum	1	0		0	None	wild	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Eastern grey kangaroo	25	23		0	None	wild	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Eastern grey kangaroo	25	23		0	None	wild	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Swamp wallaby	1	1		0	None	wild	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Swamp wallaby	1	1		0	None	wild	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Brush-tailed rock wallaby	4	0		0	None	not clear	Victoria
Stalder	2015	sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Brush-tailed rock wallaby	4	0		0	None	not clear	Victoria

Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Tammar wallaby	4	0		0	None	captive	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Tammar wallaby	4	0		0	None	captive	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Yellow-footed rock wallaby	1	0		0	None	captive	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Yellow-footed rock wallaby	1	0		0	None	captive	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Koala	8	0		0	None	Not clear	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Koala	8	0		0	None	not clear	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Tasmanian devil	21	1		0	None	not clear	Tasmania
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Tasmanian devil	21	1		0	None	not clear	Tasmania
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Common wombat	15	10		0	None	Wild	Victoria
Stalder	2015	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Common wombat	15	10		0	None	Wild	Victoria
Fiebig	2015	Sera	Virus neutralisation	Antibody	Koala retrovirus	Retroviridae	Koala	9	9		0	None	Wild	Queensland
Fiebig	2015	Sera	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	9	9		0	None	Wild	Queensland
Simmons	2014	Tissues	PCR	Virus (nucleic acid)	Retrovirus	Retroviridae	Brushtail possum	4	4		0	None	Wild	Queensland
Simmons	2014	Tissues	PCR	virus (nucleic acid)	Retrovirus	Retroviridae	Ringtail possum	1	1		0	None	Wild	Queensland
Simmons	2014	Tissues	PCR	virus (nucleic acid)	Retrovirus	Retroviridae	Sugar glider	2	2		0	None	Wild	Queensland
Simmons	2014	Blood	PCR	virus (nucleic acid)	Retrovirus	Retroviridae	Common wombat	5	0		0	None	Wild	Queensland
Simmons	2014	DNA	PCR	virus (nucleic acid)	Retrovirus	Retroviridae	Tasmanian devil	1	0		0	None	Wild	Queensland
Simmons	2014	Tissue	PCR	virus (nucleic acid)	Retrovirus	Retroviridae	Red necked wallaby	4	0		0	None	Wild	Queensland

Simmons	2014	Tissue	PCR	virus (nucleic acid)	Retrovirus	Retroviridae	Stripe faced dunnart	5	0		0	None	Wild	Queensland
Potter	2014	Blood	Virus neutralisation	Antibody	Ross river virus	Togaviridae	Western grey kangaroo	2632	1155		0	None	Wild	Western Australia
Hobbs	2014	Tissue	NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	1	1		0	Chlamydial disease	Wild	Queensland
Hobbs	2014	Tissue	NGS	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	1	1		0	None	Captive	Queensland
Amery-Gale	2014	Tissues	PCR	virus (nucleic acid)	Dasyurid herpesvirus 1	Herpesviridae	Yellow-footed antechinus and agile antechinus	2	2	1+/1	0	Weakness, poor body condition, death of agile antechinus	Wild	Victoria
Vaz	2013	Nasal swab	PCR and virus isolation	Virus (nucleic acid and CPE)	Macropod herpesvirus 4	Herpesviridae	Eastern grey kangaroo	1	1		0	None	Wild	Victoria
Pacioni	2013	Blood	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Woylie	200	0		0	None	Wild	Western Australia
Pacioni	2013	Blood	Virus neutralisation	Antibody	encephalomyocarditis virus (Cardiovirus A)	Picornaviridae	Woylie	66	0		0	None	Wild	Western Australia
Pacioni	2013	Blood	Virus neutralisation	Antibody	Wallal virus	Sedoreoviridae	Woylie	38	0		0	None	Wild	Western Australia
Pacioni	2013	Blood	Virus neutralisation	Antibody	Warrego virus	Sedoreoviridae	Woylie	44	0		0	None	Wild	Western Australia
Pacioni	2013	blood	Virus neutralisation	Antibody	Ross river virus	Togaviridae	Woylie	73	0		0	None	Wild	Western Australia
Pacioni	2013	Blood	Virus neutralisation	Antibody	Barmah Forest virus	Togaviridae	Woylie	72	0		0	None	Wild	Western Australia
Pacioni	2013	Blood	ELISA	Antibody	flavivirus	Flaviviridae	Woylie	73	0		0	None	Wild	Western Australia
Ávila-Arcos	2013	Skin	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	29	17		0	None	Unknown	not clear
Vogelnest	2012	Tissue	Histopathology and electron microscopy	Virus (inclusion body,	poxvirus	Poxviridae	Ringtail possum	1	1	1+/1	0	Raised nodular exophytic lesions	Captive	New South Wales

				viral particle)										
Vaz	2012	Tissues	PCR	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	3	1		0	None	Wild	Victoria
Simmons	2012	Blood, tissues	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	277	277		0	Unknown	Wild	Queensland
Simmons	2012	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	100	100		0	Unknown	Wild	New South Wales
Simmons	2012	Blood, tissues	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	169	87		0	Unknown	Wild	Victoria
Simmons	2012	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	162	24		0	Unknown	Wild	South Australia
Rose	2012	Tissues	cell culture and PCR	virus (CPE and nucleic acid)	Eubenangee virus	Sedoreoviridae	Tammar wallaby	13	11	11+/11	11	Sudden death, lethargy, neurological signs	Captive	New South Wales
Rose	2012	Cerebrospinal fluid	cell culture and PCR	Virus (CPE and nucleic acid)	Eubenangee virus	Sedoreoviridae	Tammar wallaby	1	1	1+/1	1	Sudden death, depression and recumbency	Captive	Queensland
Rose	2012	Sera	Virus neutralisation	Antibody	Encephalomyocarditis virus (Cardiovirus A)	Picornaviridae	Tammar wallaby	5	0		0	None	Captive	New South Wales
Rose	2012	Sera	Virus neutralisation	Antibody	Eubenangee virus	Sedoreoviridae	Tammar wallaby	36	0		0	None	Captive	New South Wales
Rose	2012	Sera	Virus neutralisation	Antibody	Eubenangee virus	Sedoreoviridae	Parma wallaby	3	0		0	None	Captive	New South Wales
Rose	2012	Sera	Virus neutralisation	Antibody	Eubenangee virus	Sedoreoviridae	Rock wallaby	2	0		0	None	Captive	New South Wales
Rose	2012	Sera	Virus neutralisation	Antibody	Eubenangee virus	Sedoreoviridae	Red kangaroo	2	0		0	None	Captive	New South Wales
Rose	2012	Sera	Virus neutralisation	Antibody	Eubenangee virus	Sedoreoviridae	Red-necked pademelon	1	0		0	None	Captive	New South Wales
Wilcox	2011	Ocular, nasal and oropharyngeal swabs	Virus isolation and PCR	Virus (nucleic acid)	Macropod herpesvirus 3	Herpesviridae	Eastern grey kangaroo	1	1	1+/1	0	Bilateral serous ocular and nasal discharges	Wild	Victoria

Vaz	2011	Ocular swab and tissues	PCR	Virus (nucleic acid)	Phascolarctid gammaherpesvirus 1	Herpesviridae	Koala	3	3	3+/3	0	Dermatitis, nephritis, cystitis, weakness, conjunctivitis	Wild	Victoria
Bennett	2010	lesion biopsy	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Woylie	1	1	1+/1	0	Papillomatous lesions	Wild	Western Australia
Hill	2009	Sera	ELISA	Antibody	Ross river virus	Togaviridae	Brushtail possum	82	0		0	None	Wild	New South Wales
Woolford	2008	Biopsies and tissues	Immunohistochemistry, Gross-, histopathology, electron microscopy	Virus (antigen), viral particles, intranuclear inclusion	Bandicoot papillomatosis carcinomatosis virus type 1	Papillomaviridae	Western barred bandicoot	14	3	3+/14	0	Cutaneous papilloma, carcinomas	Captive	Western Australia
Woolford	2007	Tissues and skin swabs	PCR	Virus (DNA)	Bandicoot papillomatosis carcinomatosis virus type 1	Papillomaviridae	Western barred bandicoot	44	2	2+/2	0	Cutaneous papilloma, carcinomas	Wild	Western Australia
Woolford	2007	Tissues and skin swabs	PCR	Virus (DNA)	Bandicoot papillomatosis carcinomatosis virus type 1	Papillomaviridae	Western barred bandicoot	63	28	28+/38	0	Cutaneous papilloma, carcinomas	Captive	Western Australia
Bennett	2008	tissues	PCR	Virus (nucleic acid)	Bandicoot papillomatosis carcinomatosis virus type 2	Papillomaviridae	Southern brown bandicoot	1	1	1+/1	0	Multifocal to coalescing flat alopecic skin plaques	Unknown	Western Australia
Kay	2007	Sera	Virus neutralisation	Antibody	Ross river virus	Togaviridae	Brushtail possum	102	18		0	None	Wild	Queensland
Kay	2007	Sera	Virus neutralisation	Antibody	Barmah Forest virus	Togaviridae	Brushtail possum	102	11		0	None	Wild	Queensland
Tarlinton	2006	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	98	98		0	None	Wild	Queensland
Tarlinton	2006	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	22	8		0	None	Wild	Victoria
Tarlinton	2006	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	26	0		0	None	Wild	South Australia
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Koala	72	10	1+/1	0	Sebaceous gland hyperplasia	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Eastern grey kangaroo	23	1		0	None	Captive	Queensland

Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Red kangaroo	12	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Red-necked wallaby	3	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Swamp wallaby	3	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Whiptail wallabies	5	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Greater bilby	1	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Red-legged pademelon	2	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Ringtail possum	1	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Brushtail possum	4	0		0	None	Captive	Queensland
Antonsson	2006	Skin swab	PCR	Virus (nucleic acid)	Papillomavirus	Papillomaviridae	Common wombat	1	0		0	None	Captive	Queensland
Tarlinton	2005	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	125	125	3+/3	0	Neoplasia (leukaemia or lymphoma)	Captive	Queensland
Tarlinton	2005	Blood	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	27	27	1+/1	0	Neoplasia (leukaemia or lymphoma)	Wild	Queensland
Old	2005	Sera	ELISA	Antibody	Ross river virus	Togaviridae	Tammar wallaby	224	34		0	None	Captive	New South Wales

Old	2005	Sera	ELISA	Antibody	Ross river virus	Togaviridae	Southern hairy-nosed wombat	2	0		0	None	Captive	New South Wales
Old	2005	Sera	ELISA	Antibody	Ross river virus	Togaviridae	Koala	12	0		0	None	Captive	New South Wales
Old	2005	Sera	ELISA	Antibody	Ross river virus	Togaviridae	Parma wallaby	5	0		0	None	Captive	New South Wales
Old	2005	Sera	ELISA	Antibody	Ross river virus	Togaviridae	Wallaroos	11	4		0	None	Captive	New South Wales
Johansen	2005	Sera	Virus neutralisation	Antibody	Barmah Forest virus	Togaviridae	Western native cat	6	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Barmah Forest virus	Togaviridae	Western grey kangaroo	128	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Barmah Forest virus	Togaviridae	Western brush wallaby	1	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Barmah Forest virus	Togaviridae	Quokka	62	2		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Barmah Forest virus	Togaviridae	Brushtail possum	3	0		0	None	Wild	Western Australia
Johansen	2005	sera	Virus neutralisation	Antibody	Sindbis virus	Togaviridae	Western native cat	6	1		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Sindbis virus	Togaviridae	Western grey kangaroo	128	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Sindbis virus	Togaviridae	Western brush wallaby	1	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Sindbis virus	Togaviridae	Quokka	62	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Sindbis virus	Togaviridae	Brushtail possum	3	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Trubanaman	Peribunyaviridae	Western native cat	6	0		0	None	Wild	Western Australia

Johansen	2005	Sera	Virus neutralisation	Antibody	Trubanaman	Peribunyaviridae	Western grey kangaroo	128	27		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Trubanaman	Peribunyaviridae	Western brush wallaby	1	0		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Trubanaman	Peribunyaviridae	Quokka	62	1		0	None	Wild	Western Australia
Johansen	2005	Sera	Virus neutralisation	Antibody	Trubanaman	Peribunyaviridae	Brushtail possum	3	0		0	None	Wild	Western Australia
Hanger	2000	blood and tissues	PCR	Virus (nucleic acid)	Koala retrovirus	Retroviridae	Koala	17	17		0	None	Unknown	Queensland
Reddacliff	1997	Tissues	Gross-, histo-pathology, virus isolation	Virus (CPE)	Encephalomyocarditis virus (Cardiovirus A)	Picornaviridae	Goodfellow's tree kangaroo	2	2	2+/2	2	Sudden death	Captive	New South Wales
Reddacliff	1997	Sera	Virus neutralisation	Antibody	Encephalomyocarditis virus (Cardiovirus A)	Picornaviridae	Red-necked wallaby	1	0		2	None	Captive	New South Wales
Reddacliff	1997	Sera	Virus neutralisation	Antibody	Encephalomyocarditis virus (Cardiovirus A)	Picornaviridae	Red kangaroo	1	0		0	None	Captive	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	Eastern grey kangaroo	48	21		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	Brown antechinus	23	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	Dusky antechinus	14	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	White-footed dunnart	3	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	Southern brown bandicoot	4	1		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	Long-nosed bandicoot	8	2		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	Red-necked wallaby	7	6		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Ross river virus	Togaviridae	Swamp wallaby	6	5		0	None	Wild	New South Wales

Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	Eastern grey kangaroo	25	1		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	Brown antechinus	8	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	Dusky antechinus	2	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	White-footed dunnart	2	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	Southern brown bandicoot	5	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	Long-nosed bandicoot	4	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	Red-necked wallaby	5	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Barmah Forest virus	Togaviridae	Swamp wallaby	4	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	Eastern grey kangaroo	6	3		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	Brown antechinus	6	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	Dusky antechinus	2	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	White-footed dunnart	2	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	Southern brown bandicoot	4	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	Long-nosed bandicoot	5	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	Red-necked wallaby	3	1		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Gan Gan virus	Peribunyaviridae	Swamp wallaby	3	0		0	None	Wild	New South Wales

Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	Eastern grey kangaroo	7	5		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	Brown antechinus	11	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	Dusky antechinus	2	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	White-footed dunnart	2	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	Southern brown bandicoot	4	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	Long-nosed bandicoot	4	0		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	Red-necked wallaby	3	1		0	None	Wild	New South Wales
Vale	1991	Blood	Microneutralisation test	Antibody	Trubanaman virus	Peribunyaviridae	Swamp wallaby	3	0		0	None	Wild	New South Wales
Rothwell	1984	Tissue	histopathology and electron microscopy	Virus (inclusion body, viral particle)	Western grey kangaroopox virus	Poxviridae	Western grey kangaroo	1	1	1+/1	0	Papillomatous lesions	Unknown	New South Wales
Cybinski	1982	Sera	Virus neutralisation	Antibody	*Palyam virus*	Sedoreoviridae	Kangaroo	102	0		0	None	Unknown	Australia (unknown state)
Cybinski	1982	Sera	Virus neutralisation	Antibody	*Palyam virus*	Sedoreoviridae	Possum	41	0		0	None	Unknown	Australia (unknown state)
Cybinski	1982	Sera	Virus neutralisation	Antibody	*Palyam virus*	Sedoreoviridae	Wallaby	9	0		0	None	Unknown	Australia (unknown state)
Wilks	1981	Tissues	Histopathology and electron microscopy	Virus (CPE and viral particles)	Macropod herpesvirus 2	Herpesviridae	Dorcopsis wallaby	14	10	10+/10	10	Moribund and death	Captive	Victoria
Wilks	1981	Tissues	histopathology and electron microscopy	Virus (CPE and	Macropod herpesvirus 2	Herpesviridae	Quokka	1	1	1+/1	1	Conjunctivitis, death	Captive	Victoria

				viral particles)										
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Red kangaroo	9	9		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Eastern grey kangaroo	5	5		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Western grey kangaroo	2	2		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Parma wallaby	2	0		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Tammar wallaby	22	1		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	red-necked wallaby	2	0		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Tasmanian red-necked wallaby	2	1		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Goodfellow's tree kangaroo	1	1		0	None	Captive	Victoria
Wilks	1981	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 2	Herpesviridae	Dorcopsis wallaby	2	2		0	None	Captive	Victoria
Callinan	1981	Tissues	histopathology and virus isolation	Virus (intranuclear inclusion and CPE	Macropod herpesvirus 2	Herpesviridae	Grey dorcopsis wallaby	8	8	8+/8	8	Death	Captive	Victoria
Callinan	1981	Tissues	Histopathologyy and virus isolation	Virus (intranuclear inclusion and CPE	Macropod herpesvirus 2	Herpesviridae	Quokka	1	1	1+/1	1	Death	Captive	Victoria
Callinan	1981	Tissues	Histopathology	Virus (intranuclear inclusion)	Herpesvirus	Herpesviridae	Western grey kangaroo	1	1	1+/1	0	Dehydration, dyspnoea, death	Captive	Victoria
Dickson	1980	Tissues	Gross-, histo-pathology, virus isolation, electron microscopy	Virus (intranuclear inclusion, CPE and viral particles)	Macropod herpesvirus 2	Herpesviridae	Woylie	8	8	8+/8	8	Death	Captive	Western Australia

Dickson	1980	Tissues	Gross-, histo-pathology, virus isolation, electron microscopy	Virus (intranuclear inclusion, CPE and viral particles)	Macropod herpesvirus 2	Herpesviridae	Rufous rat kangaroo	11	11	11+/11	11	Death	Captive	Western Australia
Finnie	1976	Tissues	Gross-, histo-pathology, virus isolation, electron microscopy	Virus (intranuclear inclusion, CPE and viral particles)	Macropod herpesvirus 1	Herpesviridae	Parma wallaby	20	16	16+/16	not specific	Respiratory rales, conjunctivitis, incoordination, moribund and death	Captive	New South Wales
Papadimitriou	1972	Tissues	Histopathology and electron microscopy	Virus (inclusion body, viral particle)	Poxvirus	Poxviridae	Quokka	1	1	1+/1	0	Epidermal papillomata	Wild	Western Australia
Doherty	1971	Blood	Virus isolation	virus (CPE)	Ross river virus	Togaviridae	Agile wallaby	2	17		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Ross river virus	Togaviridae	Agile wallaby	144	169		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Ross river virus	Togaviridae	Eastern grey kangaroo	28	25		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Sindbis virus	Togaviridae	Agile wallaby	60	141		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Sindbis virus	Togaviridae	Eastern grey kangaroo	9	18		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Getah virus	Togaviridae	Agile wallaby	64	134		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Getah virus	Togaviridae	Eastern grey kangaroo	6	21		0	None	Wild	Queensland
Doherty	1971	blood	Haemagglutination -inhibition,	antibody	Murray valley encephalitis virus	Flaviviridae	Agile wallaby	157	169		0	None	Wild	Queensland

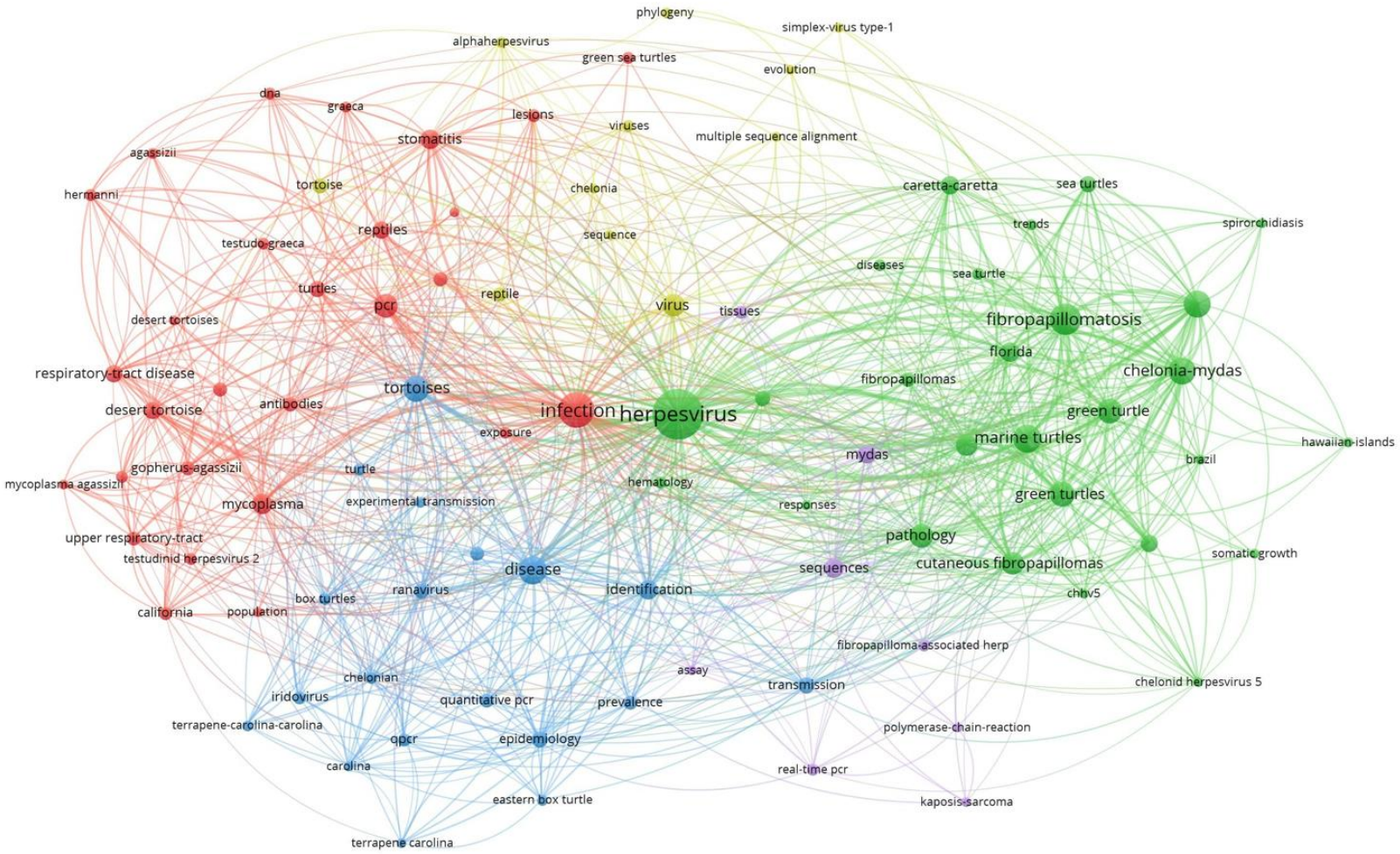
			virus neutralization											
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Murray valley encephalitis virus	Flaviviridae	Eastern grey kangaroo	24	28		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Kokobera virus	Flaviviridae	Agile wallaby	17	18		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Kokobera virus	Flaviviridae	Eastern grey kangaroo	8	10		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Edge Hill virus	Flaviviridae	Agile wallaby	15	18		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Edge Hill virus	Flaviviridae	Eastern grey kangaroo	10	10		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	West Nile virus (kunjin strain)	Flaviviridae	Agile wallaby	15	18		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	West Nile virus (kunjin strain)	Flaviviridae	Eastern grey kangaroo	8	10		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Koongol virus	Peribunyaviridae	Eastern grey kangaroo	5	1		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Wongal virus	Peribunyaviridae	Eastern grey kangaroo	5	0		0	None	Wild	Queensland
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Koongol virus	Peribunyaviridae	Agile wallaby	46	3		0	None	Wild	Queensland
Webber	1978	Sera	Serum neutralization	Antibody	Macropod herpesvirus 1	Herpesviridae	Quokka	2	0		0	None	Captive	not clear
Doherty	1971	Blood	Haemagglutination -inhibition, virus neutralization	Antibody	Wongal virus	Peribunyaviridae	Agile wallaby	46	2		0	None	Wild	Queensland

Stanley	1963	Sera	Haemagglutination -inhibiton	Antibody	mammalian orthoreovirus	Spinareoviridae	Kangaroo	15	11		0	None	Wild	Western Australia
Stanley	1963	Sera	Haemagglutination -inhibition	Antibody	mammalian orthoreovirus	Spinareoviridae	quokka	72	47		0	None	Wild	Western Australia
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Parma wallaby	13	4		0	None	Captive	not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Parma wallaby	13	0		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Parma and tammar wallabies	2	1		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Eastern grey kangaroo	7	5		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Eastern grey kangaroo	1	0		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Tammar wallaby	43	15		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Rock wallabies	102	20		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Rock wallabies	4	4		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Red-necked and red-legged pademelon	7	2		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Spectacled hare-wallaby	2	2		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Hill wallaroos	18	16		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Hill wallaroos	17	3		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Red-necked wallaby	6	0		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Red kangaroos	2	0		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Red kangaroos	6	5		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Long-nosed potoroos	13	3		0	None	Wild	Not clear

Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Short-nosed and long-nosed bandicoots	17	5		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Common brushtail possums	10	0		0	None	Wild	Not clear
Webber	1978	sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Common brushtail possums	2	0		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Common brushtail possums	10	0		0	None	Wild	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Common brushtail possums	21	1		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Crest-tailed mulgara	1	1		0	None	Captive	Not clear
Webber	1978	Sera	Virus neutralisation	Antibody	Macropod herpesvirus 1	Herpesviridae	Fat-tailed dunnart	1	0		0	None	Captive	Not clear

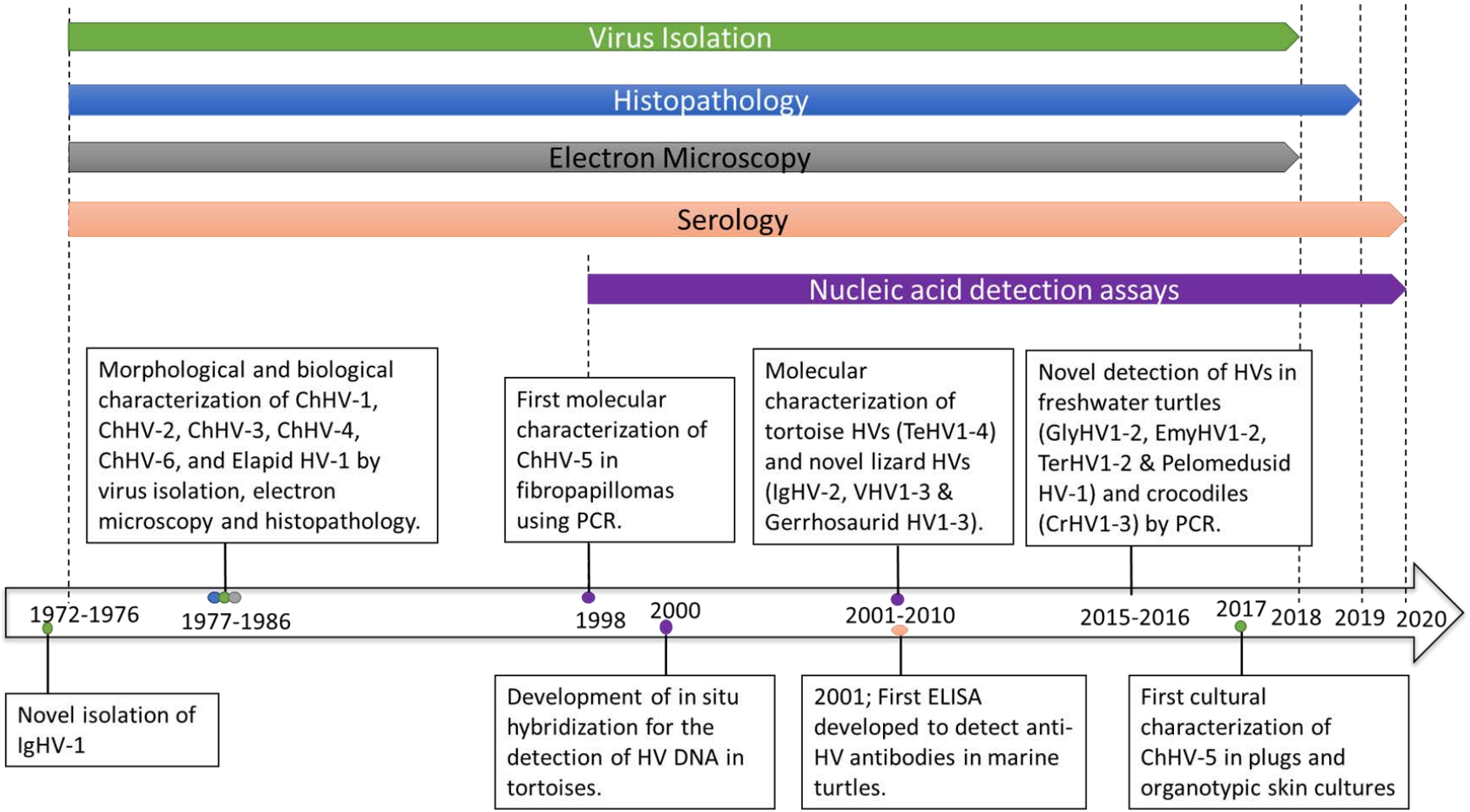
Supplementary Table 2. 4. Determination of an association between viral infection and clinical signs using odds ratio.

Virus	Observed clinical sign	Status	Positive	Negative	Odds ratio (95 % CI)	Significance level (P)
Retrovirus	Neoplasia	Sick	61	1	22.6088 (3.1304 to 163.2875)	0.0020
		Healthy	3065	1135		
Papillomavirus	Papillomas/carcinomas	Sick	36	21	31.3714 (13.6311 to 72.2002)	<0.001
		Healthy	10	183		
Picornavirus	Sudden death and necrotizing nonsuppurative myocarditis	Healthy	2	0	27.2581 (1.2473 to 595.6710)	0.0357
		Sick	15	84		
Arterivirus	Sudden death, tachypnoea, moribundity, blindness,	Sick	3	6	6.0500 (1.4405 to 25.4097)	0.00140
		Healthy	30	357		



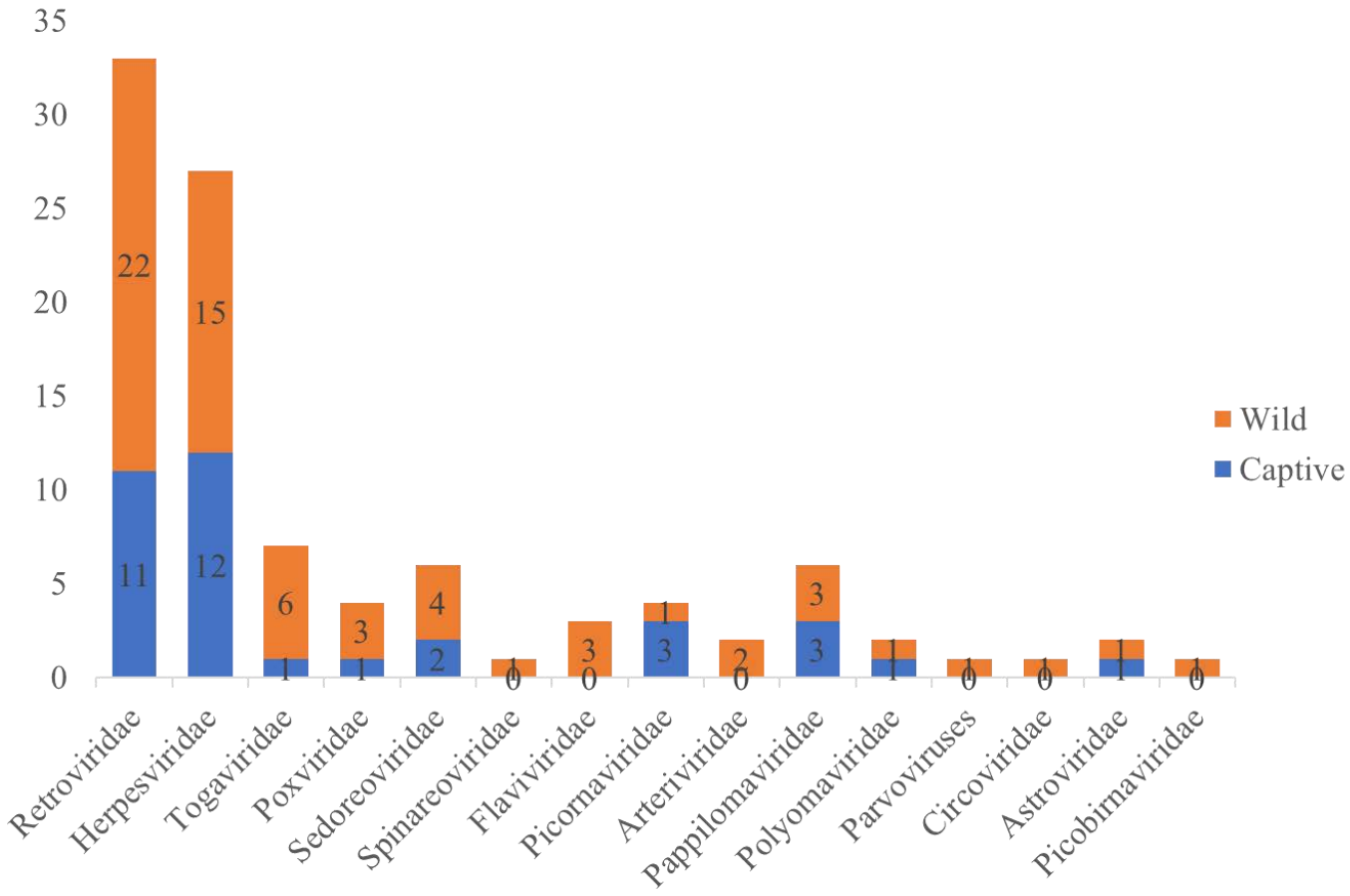
Supplementary Figure 2. 1. Co-occurrence map of all keywords from 1975 to 2021. We found a total of 93 keywords with a minimum of 5 occurrences. Node size represents the number of occurrences of each keyword. The lines denote the total link strength between keywords, and it

is proportional with the width. The line colours denote five different keyword clusters. The clusters gave an indication of the main areas research interest of reptilian HVs.



Supplementary Figure 2. 2. A timeline of reptilian HV diagnosis. A review of articles conducted from 1972 to 2020 showed that VI, EM, histopathology, and serologic assays have been used for the detection of reptilian HVs since 1972. Nucleic acid detection assay (PCR) was first

reported in 1998 for detection of ChHV-5 in fibropapillomas. Events were chosen based on information from the extracted articles (Supplementary Table 2.2).



Supplementary Figure 2. 3. An overview of the number of viral investigations in captive and wild marsupials.

Appendix Two: Supplementary Files from Chapter Three

Supplementary Table 3. 1. List of GenBank accession numbers for nucleotide sequences used for the design of consensus primers.

Alphaherpesvirinae

Varanid herpesvirus 2 (AB189433)

Varanid herpesvirus 1 (AY437559)

Tortoise alphaherpesvirus 4 (GQ222415)

Tortoise alphaherpesvirus 2 (AY916792)

Testudinid herpesvirus 3 (NC_027916)

Terrapene herpesvirus 1 (KJ004665)

Suid herpesvirus 1 (NC_006151)

Psittacid herpesvirus 1 (NC_005264)

Phocid alphaherpesvirus 1 (MH509440)

Passerid herpesvirus 1 (DQ287313)

Meleagrid herpesvirus 1 (NC_002641)

Macropodid herpesvirus 1 (NC_029132)

Macacine herpesvirus 1 (NC_004812.1)

Loggerhead orocutaneous herpesvirus (EU004542)

Loggerhead genital-respiratory herpesvirus (EU004539)

Lacerta viridis herpesvirus 1 (EU527336)

Iguanid herpesvirus 2 (AY236869)

Human herpesvirus 3 (KU529562.1)

Human herpesvirus 2 (AY038366.1)

Human herpesvirus 1 (MH697533.1)

Gerrhosaurid herpesvirus 3 (AF416630)

Gerrhosaurid herpesvirus 2 (AF416628)

Gerrhosaurid herpesvirus 1 (AF416629)

Gaviid herpesvirus 1 (GU130289)

Gallid herpesvirus 3 (NC_002577.1)

Gallid herpesvirus 2 (NC_002229.3)

Gallid alphaherpesvirus 1 (MF156851)

Equid herpesvirus 9 (NC_011644.1)

Equid herpesvirus 8 (NC_017826)

Equid herpesvirus 4 (KF434386.1)

Equid herpesvirus 3 (NC_024771)

Equid herpesvirus 1 (KF434381.1)

Emydoidea herpesvirus 1 (KY849379)

Emydid herpesvirus 1 (KF478668)

Duck enteritis virus (EF643560.1)

Columbid herpesvirus 1 (KJ995972.1)

Chelonid alphaherpesvirus 6 (EU006876)

Chelonid alphaherpesvirus 5 (NC_028891)

Bovine herpesvirus 5 (NC_005261.1)

Bovine herpesvirus 2 (MN086791.1)

Bovine herpesvirus 1.2 (KM258881.1)

Betaherpesvirinae

Macaca nemestrina herpesvirus 7 (NC_030200)

Porcine cytomegalovirus strain BJ09 (NC_022233)

Elephantid herpesvirus 1 (NC_020474)

Caviid herpesvirus 2 strain 21222 (NC_020231)

Murid herpesvirus 8 (NC_019559)

Saimiriine herpesvirus 4 strain SqSHV (NC_016448)

Aotine herpesvirus 1 strain S34E (NC_016447)

Cercopithecine herpesvirus 5 strain 2715 (NC_012783)

Human herpesvirus 5 strain Merlin (NC_006273)

Macacine herpesvirus 3 (NC_006150)

Tupaiid herpesvirus 1 (NC_002794)

Rat cytomegalovirus Maastricht (NC_002512)

Human herpesvirus 7 (NC_001716)

Human betaherpesvirus 6A (NC_001664)

Human herpesvirus 6B (NC_000898)

Gammaherpesvirinae

Suid gammaherpesvirus 4 (NC_038265)

Suid gammaherpesvirus 3 (NC_038264)

Saimiriine gammaherpesvirus 2 (NC_001350)

Ovine gammaherpesvirus 2 (NC_007646)

Murid gammaherpesvirus 4 (NC_001826)

Macacine herpesvirus 4 (NC_006146)

Macacine gammaherpesvirus 5 (NC_003401)

Human gammaherpesvirus 8 (NC_009333)

Human gammaherpesvirus 4 (NC_007605)

Felid gammaherpesvirus 1 (NC_028099)

Equid gammaherpesvirus 5 (NC_026421 - ORF9)

Equid gammaherpesvirus 2 (NC_001650)

Callitrichine gammaherpesvirus 3 (NC_004367)

Bovine gammaherpesvirus 6 (NC_024303)

Bovine gammaherpesvirus 4 (NC_002665)

Ateline gammaherpesvirus 3 (NC_001987)

Alcelaphine gammaherpesvirus 2 (NC_024382)

Alcelaphine gammaherpesvirus 1 (NC_002531)

Supplementary Table 3. 2. list of all the primers tested in this chapter. This table was grouped according to the different primer chemistry (CODEHOP, Degenerate and LNA incorporated).

S/N	Primer Name	Primer Sequence (5′→3′)	Orientation	Primer Type	Target Gene	HV-subfamily
1	DIECT-F1-12X	AATGTGCTTTgayathgartg	Forward	CODEHOP	DPOL	α-HV
2	GYNI-R-48X	CAAAGTTTACdatrttrtancc	Reverse	CODEHOP	DPOL	α-HV
3	NFDW-F2-8x	CATCGTCaayttygaytg	Forward	CODEHOP	DPOL	α-HV
4	GEYC-R2-32x	AGTCCTGGATrcartaytcncc	Reverse	CODEHOP	DPOL	α-HV
5	YGDT-F1-16x	GCGAGTAATTtayggngayac	Forward	CODEHOP	DPOL	α-HV
6	ECEK-R-16X	TAGTAAAGGTyttytcrcaytc	Reverse	CODEHOP	DPOL	α-HV
7	QQIR-F 24x	CTTCGACGGCcarcarathmg	Forward	CODEHOP	DPOL	α-HV
8	DWLA-R 64x	TGCGCATngcnarccartc	Reverse	CODEHOP	DPOL	α-HV
9	YGDT-F2 16x	GCGCGTCATCtayggngayac	Forward	CODEHOP	DPOL	α-HV
10	EKTF-R 32x	GCAGTTTGGTraangtyttytc	Reverse	CODEHOP	DPOL	α-HV
11	AlphaFWD1	AGCATHATYCAGGCBCAYAAYCTSTGYTTYA	Forward	Degenerate	DPOL	α-HV
12	AlphaREV1	CYTTRATSGCVRSCTGYTGYT	Reverse	Degenerate	DPOL	α-HV
13	AlphaFWD2	CHAGCATHATYCAGGCBCAYAAY	Forward	Degenerate	DPOL	α-HV
14	AlphaREV2	TTRATBGCVRVCTGYTGYTTRTC	Reverse	Degenerate	DPOL	α-HV
15	AlphaFWD3	GAYTTYGCYAGCYTRTAYC	Forward	Degenerate	DPOL	α-HV
16	BetaFWD1	TGYAAYKCBKTBTAYGGBKTYAC	Forward	Degenerate	DPOL	β-HV
17	BetaREV1	CKVACBARWTCYAYVCCYTTMAW	Reverse	Degenerate	DPOL	β-HV
18	BetaFWD2	YAAYKCBKTBTAYGGBKTYACSGG	Forward	Degenerate	DPOL	β-HV
19	BetaREV2	AYRAAVAYGCTRTCSGTRTC	Reverse	Degenerate	DPOL	β-HV
20	BetaFWD3	GAYACBGAYAGYVTBTTYRT	Forward	Degenerate	DPOL	β-HV
21	BetaREV3	CCNAYRTANCKYTTYTTRC	Reverse	Degenerate	DPOL	β-HV
22	BetaFWD4	TDCCVTGYBTNSCNVTNGC	Forward	Degenerate	DPOL	β-HV
23	BetaREV4	ARVABYTTHTCDAMYTCNA	Reverse	Degenerate	DPOL	β-HV

24	BetaFWD5	GTNTTYGAYTTYGCMAGYY	Forward	Degenerate	DPOL	β-HV
25	BetaREV5	TTDAGHGCMAGCTGYTBYTTRTC	Reverse	Degenerate	DPOL	β-HV
26	BetaFWD6	ATHATSATGGCHMAYAAYCTSTGYTAYW	Forward	Degenerate	DPOL	β-HV
27	BetaFWD7	GAYTTYGCMAGYYTSTAYC	Forward	Degenerate	DPOL	β-HV
28	BetaFWD_gb1	GARGCBTGGTGTHWVGATCA	Forward	Degenerate	gB	β-HV
29	BetaREV_gb2	CCNARYARRATYTCRTTRT	Reverse	Degenerate	gB	β-HV
30	BetaFWD_gb2	AYAAYGARATYYTRYTNGG	Forward	Degenerate	gB	β-HV
31	BetaFWD_HE1	GTSTTYTTYTAYTRSTTYTAYAACGC	Forward	Degenerate	helicase	β-HV
32	BetaREV_He1b	CTTRTTRTTRATRAASATBAYCCAGTT	Reverse	Degenerate	helicase	β-HV
33	BetaFWD_HE2	GYGTSGGBTCKCCBACGCAGAC	Forward	Degenerate	helicase	β-HV
34	BetaREV_He2	CVACRTASKCNAYRTGCTC	Reverse	Degenerate	helicase	β-HV
35	BetaFWD_HE3	CKCCBACGCAGACSGAGGC	Forward	Degenerate	helicase	β-HV
36	BetaFWD_He4	AMBTAYACKCTSGARGGNTAYAC	Forward	Degenerate	helicase	β-HV
37	BetaREV_He4	GACATSSCMACGTARATSTGRCTCA	Reverse	Degenerate	helicase	β-HV
38	BetaREV_He5	CCYTGRCTYTTRGYGATSGTCAT	Reverse	Degenerate	helicase	β-HV
39	BetaREV_He6	TCSCCRAARTCKAYNGCCAC	Reverse	Degenerate	helicase	β-HV
40	GammaFWD1	GGVTAYAACRTNKSMAAYTTTGA	Forward	Degenerate	DPOL	γ-HV
41	GammaREV1	GGRTASAGGCTRGCAAARTC	Reverse	Degenerate	DPOL	γ-HV
42	GammaFWD2	GVCARCARATHMGVGTBTT	Forward	Degenerate	DPOL	γ-HV
43	GammaREV2	CABAGRTTRTGNGCYTGDATRATRCTBGG	Reverse	Degenerate	DPOL	γ-HV
44	GammaFWD3	GAYTTTGCBAGYYTBTAYC	Forward	Degenerate	DPOL	γ-HV
45	GammaREV3	GTVMMYTTRATDGCHAGYTGYTG	Reverse	Degenerate	DPOL	γ-HV
46	GammaFWD4	TVRTNTAYGGNGAYACNGAY	Forward	Degenerate	DPOL	γ-HV
47	GammaREV4	CKNAYNARVTCNACVCCYTTCA	Reverse	Degenerate	DPOL	γ-HV
48	GammaREV5	AKDGTRGARTARCABARRTTRTKNGCYTG	Reverse	Degenerate	DPOL	γ-HV
49	GammaREV6	TTRATRGCMAGCTGCTGYTTRTC	Reverse	Degenerate	DPOL	γ-HV
50	BetaREV_gb1	YT[+C]YARR[+T]CRAANACGTT	Reverse	LNA incorporated	gB	β-HV
51	BetaFWD3.LNA	GAY[+A]CB[+G]AY[+A]GYV[+T]BTTYRT	Forward	LNA incorporated	DPOL	β-HV

52	BetaREV3.LNA	CCN[+A]YR[+T]AN[+C]KYT[+T]YTTRC	Reverse	LNA incorporated	DPOL	β-HV
53	BetaREV4.LNA	ARV[+A]BYT[+T]HT[+C]DAMY[+T]CNA	Reverse	LNA incorporated	DPOL	β-HV
54	BetaREV_He1	CTTR[+T]TRTTRATRAASATBAYCCAGTT	Reverse	LNA incorporated	helicase	β-HV
55	BetaREV_He1c	YTTR[+T]TRTTDAYRAABATBAYCCARTT	Reverse	LNA incorporated	helicase	β-HV
56	BetaREV_He3	AGM[+G]GNADVCCRAAYTCYAT	Reverse	LNA incorporated	helicase	β-HV

CODEHOP: Consensus-DEgenerate Hybrid Oligonucleotide Primer; DPOL: DNA polymerase; gB: glycoprotein B; HV: herpesvirus; LNA: locked nucleic acid.

Supplementary Table 3. 3. Results of herpesvirus testing using different primer combinations in both singleplex and multiplex formats. The primer sets with best outcomes (bolded) were selected for this study.

Primer pair	Expected size (bp)	α-HVs																β-HVs			γ-HVs		
		HHV 1	HHV 2	MaHV1	MaHV2	PhaHV3	CoHV 1	MeHV1	GaHV 2	BoHV 1	HHV3	EHV4	FeHV1	ChHV 5	CrHV 1	CrHV2	CrHV3	HHV5	HHV6	HHV 7	HHV 4	HHV 8	PhaHV1
Singleplex combination																							
DIECT-F1-12X and GYNI-R-48X	319	NT	NT	Neg	Neg	Neg	NT	NT	NT	Neg	NT	NT	Neg	Neg	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
NFDW-F2-8x & GEYC-R2-32x	346	Pos	Pos	Neg	Neg	Neg	NT	NT	NT	Pos	Pos	Pos	Pos	Neg	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
AlphaFWD1 & AlphaREV1	265	Pos	Pos	Pos	Pos	Pos	NT	Pos	Neg	Pos	Pos	Pos	Pos	Pos	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
YGDT-F1-16x & ECEK-R-16X	157	Pos	Neg	Neg	Neg	Neg	NT	NT	NT	Pos	Neg	Pos	Neg	Neg	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
QQIR-F 24x & DWLA-R 64x	598	Pos	Pos	Pos	Pos	Pos	NT	Pos	Neg	Pos	Pos	Pos	Pos	Neg	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
YGDT-F2 16x & EKTF-R 32x	161	Pos	Pos	Neg	Neg	Neg	NT	NT	NT	Pos	Neg	Neg	Neg	Neg	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A

AlphaFWD2 & AlphaREV2	277	Pos	Pos	Pos	Pos*	Pos*	NT	Pos	Neg	Neg	Pos	Pos	Pos	Pos	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
AlphaFWD2 & DWLA-R 64x	187	Pos	Pos	Neg	Pos	Pos*	NT	Pos	Pos	Neg	Pos	Pos	Pos	Neg	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
AlphaFWD1 & DWLA-R 64x	187	Pos	Pos	Pos	Pos	Pos	NT	Pos	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	N/A	N/A	N/A	N/A	N/A	N/A
AlphaFWD2 & AlphaREV1	265	NT	NT	Pos	Neg	Neg	NT	Neg	Neg	Neg	NT	NT	NT	Neg	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
AlphaFWD3 & AlphaREV2	265	Pos	Pos	Pos	Neg	Pos	NT	Pos	Pos	Pos	Neg	Pos	Pos	Pos	NT	NT	NT	N/A	N/A	N/A	N/A	N/A	N/A
AlphaFWD1 & AlphaREV2	**265-277**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	N/A	N/A	N/A	N/A	N/A	N/A
BetaFWD1 & BetaREV1	518	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD2 & BetaREV2	309	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Pos	Neg	N/A	N/A	N/A
BetaFWD3 & BetaREV3	176	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD4 & BetaREV4	385	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD4 & BetaREV2	267	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD1 & BetaREV4	427	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A

BetaFWD2 & BetaREV3	465	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD2, BetaREV2 & BetaREV3	309-465	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD1 & BetaREV2	311	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Pos	Neg	N/A	N/A	N/A
BetaFWD2 & BetaREV1	515	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD3.LNA & BetaREV3.LNA	179	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFW4 & BetaREV4.LNA	385	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD5 & BetaREV5	301	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	Neg	N/A	N/A	N/A
BetaFWD6 & BetaREV5	264	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD7 & BetaREV5	296	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	Pos	N/A	N/A	N/A
BetaFWD_HE1 & BetaREV_He1	246	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos	Neg	N/A	N/A	N/A
BetaFWD_HE1 & BetaREV_He1c	246	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	Neg	N/A	N/A	N/A
BetaFWD_HE1 & BetaREV_He3	309	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos	Neg	N/A	N/A	N/A

BetaFWD_He4 & BetaREV_He5	338	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos	Neg	N/A	N/A	N/A
BetaFWD_gb1 & BetaREV_gb1	**564**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg
BetaFWD_HE1 & BetaREV_He1b	246	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos	Pos*†	Neg	Neg	Neg
BetaFWD_HE1 & BetaREV_He2	330	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos*	Pos*	Neg	Neg	Neg
BetaFWD_HE2 & BetaREV_He2	255	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	Pos*	N/A	N/A	N/A
BetaFWD_He2 & BetaREV_He3	229	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos*	Pos†	N/A	N/A	N/A
BetaFWD_He4 & BetaREV_He4	257	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos*	Neg	N/A	N/A	N/A
BetaFWD_He4 & BetaREV_He6	293	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	Neg	N/A	N/A	N/A
BetaFWD_gb1 & BetaREV_gb2	332	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos*	Neg	Neg	N/A	N/A	N/A
BetaFWD_HE3 & BetaREV_He2	246	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	Neg	N/A	N/A	N/A
BetaFWD_HE3 & BetaREV_He3	221	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	Neg	N/A	N/A	N/A
BetaFWD_gb2 & BetaREV_gb1	251	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos*	Neg	N/A	N/A	N/A

GammaFWD1 & GammaREV1	650	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**Pos**	**Pos**	**Pos**
GammaFWD2 & GammaREV2	205	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	NT
GammaFWD3 & GammaREV3	300	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos*	NT
GammaFWD4 & GammaREV4	233	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	NT
GammaFWD2 & GammaREV5	217	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Neg	NT
GammaFWD3 and GammaREV6	300	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Pos	Pos	Neg
GammaFWD2 & GammaREV6	423	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Neg	Neg	NT
Duplex/Multiplex combination																							
AlphaFWD1 & AlphaREV2; BetaFWD2 & BetaREV2; GammaFWD3 & GammaREV3	231-309	Pos	Pos	Pos	Pos†	Pos	NT	Pos	Pos	Pos	Pos	Pos	Pos	Pos	NT	NT	NT	Pos	Pos*	Neg	Pos	Pos	NT
AlphaFWD1 & AlphaREV2; BetaFWD3 & BetaREV3; GammaFWD1 & GammaREV1	176-641	Pos	Pos	NT	Neg	NT	NT	NT	NT	NT	Pos*	NT	NT	NT	NT	NT	NT	Neg	Neg	Pos	Pos	Neg	NT

AlphaFWD1 & AlphaREV2 BetaFWD2 & BetaREV2 BetaFWD3 & BetaREV3 GammaFWD3 & GammaREV3	176-309	Pos	NT	NT	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Neg	Neg	Pos*	Neg	NT
AlphaFWD1 &AlphaREV2 BetaFWD4 & BetaREV4; GammaFWD3 & GammaREV3	265-385	Pos	NT	NT	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos	Pos*	Neg	Pos	Pos	NT
AlphaFWD1 & AlphaREV2; BetaFWD4 & BetaREV3; GammaFWD3 & Gamma REV3	265-423	NT	NT	NT	Pos*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos*	Neg	Neg	NT	Pos	NT
AlphaFWD1 & AlphaREV2; BetaFWD3 & BetaREV4; GammaFWD3 & GammaREV3	136-300	NT	NT	NT	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos	Pos*	Neg	NT	Pos	NT
AlphaFWD1 & AlphaREV2; BetaFWD1 & BetaREV1; GammaFWD3 & Gamma REV3	265-515	NT	NT	NT	Neg	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Neg	Neg	NT	Neg	NT
AlphaFWD1 & AlphaREV2; BetaFWD3 &	176-300	NT	NT	NT	Neg	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Neg	Neg	NT	Neg	NT

BetaREV3; GammaFWD3 & Gamma REV3																							
AlphaFWD1 & AlphaREV2; BetaFWD3 & BetaREV4; GammaFWD1 & Gamma REV1	265-650	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos	Pos*	Pos	Pos	Pos	NT
AlphaFWD1 & AlphaREV2; BetaFWD3, BetaREV3 & BetaREV4; GammaFWD1 & GammaREV1	165-650	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos*	Pos*	Pos	Pos	Pos	NT
AlphaFWD1 & AlphaREV2; BetaFWD2, BetaREV2 & BetaFWD3; GammaFWD1 & Gamma REV1	179-650	Pos	NT	NT	Pos*†	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos	Pos	Pos*	NT
AlphaFWD1 & AlphaREV2 BetaFWD3.LNA & BetaREV4.LNA; GammaFWD1 & Gamma REV1	179-650	Pos	NT	NT	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos*	Pos*	Pos	Pos	Pos*	NT
AlphaFWD1 & AlphaREV2; BetaFWD3.LNA,	165-650	NT	Pos	NT	Pos	NT	NT	NT	NT	NT	Pos*	NT	NT	Pos	NT	NT	NT	Pos*	Pos*	Pos	Pos	Pos*	NT

BetaREV3.LNA & BetaREV4.LN; BetaFWD3, BetaREV3 & BetaREV4; GammaFWD1 & GammaREV1																						
AlphaFWD1 & AlphaREV2; BetaFWD2 & BetaREV2	261-265	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Pos*	Neg	Neg	Neg	NT
AlphaFWD1 & AlphaREV2; GammaFWD3 & Gamma REV3	265-309	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Pos*	Pos*	Neg	Pos	Pos*	NT
BetaFWD2, BetaREV2; GammaFWD3 & Gamma REV3	261-309	Neg	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Pos*	Neg	Pos	Neg	NT
AlphaFWD1 & AlphaREV2; BetaFWD3 & BetaREV4	179-309	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Neg	Neg	Neg	Neg	NT
AlphaFWD1 & AlphaREV2; GammaFWD1 & Gamma REV1	265-650	Pos	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Neg	Neg	Pos	Neg	NT
BetaFWD3, BetaREV4; GammaFWD1 & Gamma REV1	179-650	Neg	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Neg	Neg	Neg	Pos	Pos*	NT
AlphaFWD1 & AlphaREV2 BetaFWD7 &	265-296	Pos	NT	NT	NT	Pos	NT	NT	NT	Pos	Pos*	NT	NT	Pos	NT	NT	NT	Pos	Pos*	Pos†	Pos	Pos	NT

BetaREV5; GammaFWD1, GammaFWD3 & GammaREV6																						
AlphaFWD1 & AlphaREV2 BetaFWD_gb1 & BetaREV_gb1; GammaFWD1 & GammaFWD1	265-650	Pos	Pos	NT	Neg	NT	NT	NT	NT	NT	Neg	NT	NT	NT	NT	NT	NT	Neg	Neg	Neg	Pos	Neg	NT

HVs: herpesviruses; NT: not tested; N/A: not applicable; Pos: positive; Neg: negative.

*Faint band

†Non-specific bands present

Appendix Three: Supplementary Files from Chapter Four

Supplementary Table 4. 1. Morphometric data from freshwater turtles.

Site	**Date**	**Tag Number**	**Old Tag**	**Lesion**	**Describe lesions**	**Species**	**Age class**	**Sex**	**Weight (kg)**	**CCL (cm)**	**SCL (cm)**	**SCW (cm)**	**PL (cm)**	**PW (cm)**	**TP (cm)**	**TC (cm)**	**TV (cm)**
Alligator Creek	22/08/2020	53046	ND	Yes	Back right foot, tail	*Emydura macquarii krefftii*	Adult	Male	1.47	23.6	ND	ND	ND	ND	101.97	ND	ND
Alligator Creek	22/08/2020	53085	ND	ND	ND	*Emydura macquarii krefftii*	Adult	Male	1.33	23	ND	ND	ND	ND	98.84	ND	ND
Alligator Creek	22/08/2020	53092	ND	ND	ND	*Emydura macquarii krefftii*	Adult	ND	0.42	14.7	ND	ND	ND	ND	42.19	ND	ND
Alligator Creek	22/08/2020	53245	52835	Yes	Back left leg	*Emydura macquarii krefftii*	Adult	ND	0.75	19.3	ND	ND	ND	ND	33.08	ND	ND

Alligator Creek	22/08/2020	53251	ND	ND	ND	*Emydura macquarii krefftii*	Juvenile		0.62	17.5	ND	ND	ND	ND	42.39	ND	ND
Alligator Creek	22/08/2020	52926	ND	ND	ND	*Emydura macquarii krefftii*	Adult	Male	0.87	20.5	ND	ND	ND	ND	100.2	ND	ND
Alligator Creek	22/08/2020	53087	ND	ND	ND	*Emydura macquarii krefftii*	Adult	Male	0.935	20.6	ND	ND	ND	ND	97.72	ND	ND
Alligator Creek	22/08/2020	52906	ND	ND	ND	*Emydura macquarii krefftii*	Adult	Male	0.89	19.9	ND	ND	ND	ND	92.76	ND	ND
Alligator Creek	22/08/2020	52907		Yes	Neck	*Emydura macquarii krefftii*	Adult	Male	1	20.7	ND	ND	ND	ND	95.14	ND	ND
Alligator Creek	22/08/2020	49798	14946	ND	ND	*Emydura macquarii krefftii*	ND	Adult	1.86	24.4	ND	ND	ND	ND	60.9	ND	ND

Alligator Creek	22/08/2020	53048	ND	ND	ND	*Emydura macquarii krefftii*	Adult	Male	0.985	21.6	ND	ND	ND	ND	90.91	ND	
Alligator Creek	22/08/2020	52930	ND	ND	ND	*Emydura macquarii krefftii*	Adult		0.59	17.1	ND	ND	ND	ND	44.76	ND	
Alligator Creek	22/08/2020	53086	ND	ND	ND	*Emydura macquarii krefftii*	Adult	Male	0.835	19.7	ND	ND	ND	ND	88.98	ND	
Alligator Creek	22/08/2020	52932	ND	Yes	Back right leg	*Emydura macquarii krefftii*	Adult	Male	0.86	20.4	ND	ND	ND	ND	98.4	ND	
Alligator Creek	22/08/2020	52844	49732	ND	ND	*Emydura macquarii krefftii*	Adult	ND	0.99	21	ND	ND	ND	ND	63.31	ND	
Alligator Creek	22/08/2020	53030		Yes	Back right foot, neck	*Emydura macquarii krefftii*	Adult	ND	0.45	15.8	ND	ND	ND	ND	57.2	ND	

Alligator Creek	22/08/2020	53089	53043	ND	ND	*Emydura macquarii krefftii*	Juvenile		0.35	14.5	ND	ND	ND	ND	30.59	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Emydura macquarii krefftii*	Juvenile		0.41	15.8	ND	ND	ND	ND	31.01	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Emydura macquarii krefftii*	Juvenile		0.17	11.8	ND	ND	ND	ND	31.94	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Emydura macquarii krefftii*	Juvenile		0.365	15.7	ND	ND	ND	ND	38.48	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Emydura macquarii krefftii*	Juvenile		0.245	13.6	ND	ND	ND	ND	28.77	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Emydura macquarii krefftii*	Juvenile	ND	0.245	13	ND	ND	ND	ND	25.7	ND	

Alligator Creek	22/08/2020	ND	ND	ND	ND	*Emydura macquarii krefftii*	Juvenile	ND	0.14	11	ND	ND	ND	ND	25.52	ND	ND
Alligator Creek	22/08/2020	52931	ND	Yes	Back left leg, tail	*Myuchelys latisternum*	Adult	Female	2.025	26.1	ND	ND	ND	ND	28.45	ND	ND
Alligator Creek	22/08/2020	52904	ND	Yes	Back right thigh	*Myuchelys latisternum*	Adult	ND	0.835	19.7	ND	ND	ND	ND	54.3	ND	ND
Alligator Creek	22/08/2020	53088	ND	ND	ND	*Myuchelys latisternum*	Adult	Female	0.61	17.7	ND	ND	ND	ND	39.53	ND	ND
Alligator Creek	22/08/2020	53093	ND	ND	ND	*Myuchelys latisternum*	Juvenile	ND	0.36	14.4	ND	ND	ND	ND	39.02	ND	ND
Alligator Creek	22/08/2020	52903	ND	ND	ND	*Myuchelys latisternum*	Adult	ND	0.69	18.6	ND	ND	ND	ND	41.95	ND	ND
Alligator Creek	22/08/2020	53273	ND	Yes	Back right leg	*Myuchelys latisternum*	Adult	Female	2.445	28.5	ND	ND	ND	ND	70.36	ND	ND
Alligator Creek	22/08/2020	52929	ND	Yes	Tail	*Myuchelys latisternum*	Adult	Female	1.395	23.2	ND	ND	ND	ND	54.86	ND	ND

Alligator Creek	22/08/2020	53047	ND	ND	ND	*Myuchelys latisternum*	Adult	ND	0.875	19.3	ND	ND	ND	ND	53.3	ND	ND
Alligator Creek	22/08/2020	53045	ND	ND	ND	*Myuchelys latisternum*	Adult	ND	0.53	16.4	ND	ND	ND	ND	38.58	ND	ND
Alligator Creek	22/08/2020	53044	ND	Yes	Back right leg (2)	*Myuchelys latisternum*	Adult	ND	1.56	23.5	ND	ND	ND	ND	60.56	ND	ND
Alligator Creek	22/08/2020	53033	ND	ND	ND	*Myuchelys latisternum*	Adult	ND	1.435	24.1	ND	ND	ND	ND	59.4	ND	ND
Alligator Creek	22/08/2020	53019	ND	Yes	Tail	*Myuchelys latisternum*	Adult	ND	0.75	18.6	ND	ND	ND	ND	90.58	ND	ND
Alligator Creek	22/08/2020	53071	ND	Yes	Back left leg	*Myuchelys latisternum*	Adult	ND	0.375	14.7	ND	ND	ND	ND	32.27	ND	ND
Alligator Creek	22/08/2020	52905	ND	ND	ND	*Myuchelys latisternum*	Adult	Female	1.005	21.2	ND	ND	ND	ND	46.69	ND	ND
Alligator Creek	22/08/2020	53090	ND	ND	ND	*Myuchelys latisternum*	Juvenile	ND	0.54	17	ND	ND	ND	ND	45.83	ND	ND
Alligator Creek	22/08/2020	49711	ND	ND	ND	*Myuchelys latisternum*	Adult	Female	2.35	28.3	ND	ND	ND	ND	64.89	ND	ND

Alligator Creek	22/08/2020	53178	ND	Yes	Back right foot	*Myuchelys latisternum*	Adult	Female	1.505	25	ND	ND	ND	ND	55.34	ND	ND
Alligator Creek	22/08/2020	49706	ND	Yes	Back of neck	*Myuchelys latisternum*	Adult	Female	2.13	26.1	ND	ND	ND	ND	63.51	ND	ND
Alligator Creek	22/08/2020	52908	ND	ND	ND	*Myuchelys latisternum*	Adult	ND	0.78	19.6	ND	ND	ND	ND	47.29	ND	ND
Alligator Creek	22/08/2020	53091	ND	ND	ND	*Myuchelys latisternum*	Adult	ND	0.52	16.1	ND	ND	ND	ND	76.25	ND	ND
Alligator Creek	22/08/2020	52909	52867	Yes	Back left foot	*Myuchelys latisternum*	Adult	ND	0.303	15.6	ND	ND	ND	ND	72.25	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Myuchelys latisternum*	Juvenile	ND	0.215	13	ND	ND	ND	ND	35.97	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Myuchelys latisternum*	Juvenile	ND	0.18	11.7	ND	ND	ND	ND	25.96	ND	ND
Alligator Creek	22/08/2020	ND	ND	ND	ND	*Myuchelys latisternum*	Juvenile	ND	0.15	11.2	ND	ND	ND	ND	19.03	ND	ND
Alligator Creek	22/08/2020	52910	ND	Yes	Right arm, left	ND	Adult	ND	0.51	16.9	ND	ND	ND	ND	92.12	ND	ND

					leg, tail, stomach												
Alligator Creek	22/05/2021	ND	ND	No lesions	ND	*Myuchelys latisternum*	Juvenile	Male	0.230	13.6	12.5	10	10	5.2	6	ND	2.2
Alligator Creek	22/05/2021	52965	ND		ND	*Myuchelys latisternum*	Adult	Male	0.555	18.4	ND	13.4	14	6.8	7.6	2.4	2.4
Alligator Creek	22/05/2021	53067	ND	Yes	ND	*Myuchelys latisternum*	Adult	Male	0.495	17.4	ND	12.6	13.9	6.9	8	3.3	3.1
Alligator Creek	22/05/2021	49706, 59898	ND	Yes	ND	*Myuchelys latisternum*	Adult	Female	1.925	25.8	ND	ND	ND	ND	5.2	1.1	2.6
Alligator Creek	22/05/2021	53076	ND	Yes	ND	*Myuchelys latisternum*	Adult	Female	1.78	10.7	ND	ND	ND	ND	5.9	0.6	2.9
Alligator Creek	22/05/2021	ND	ND	No lesions	ND	*Emydura macquarii krefftii*	Juvenile		0.325	15.3	14	10.8	11.2	4.9	2.7	ND	1.1
Alligator Creek	22/05/2021	52853	ND	Yes on tail	ND	*Emydura macquarii krefftii*	Adult	Male	0.560	17.3		12.8	13.3	5.5	6	4.1	2.1

Alligator Creek	22/05/2021	52966	ND	Yes	ND	*Myuchelys latisternum*	Adult	Male	0.465	17	15.7	11.7	13	6.1	7	2.4	2.1
Alligator Creek	22/05/2021	53092	ND	No lesions	ND	*Emydura macquarii krefftii*	Juvenile	Male	0.375	15.4	14.2	11	11.9	4.2	3.7	ND	1.6
Alligator Creek	22/05/2021	53253	ND	Yes	ND	*Emydura macquarii krefftii*	Juvenile	Male	0.590	18.5	ND	12.2	14	6	3.5	ND	1.5
Alligator Creek	22/05/2021	52819, 53254	ND	No lesion	ND	*Emydura macquarii krefftii*	Adult	Male	0.540	17.1	ND	12.3	13.2	5.6	7.6	3.5	2.9

ND: No data.

Supplementary Table 4. 2. List of individual samples within each pool.

Location	**Alligator Creek**						**Ross River**				**Alligator Creek**						**Ross River**	
Pool identifier	**1 (n=28)**	**2 (n=28)**	**3 (n=9)**	**4 (n=8)**	**5 (n=9)**	**6 (n=8)**	**7 (n= 35**	**8 (n =35)**	**9 (n=12)**	**10 (n=12)**	**11 (n=30)**	**12 (n = 30)**	**13 (n=9)**	**14 (n=8)**	**15 (n=9)**	**16 (n=8)**	**17 (n = 37)**	**18 (n=35)**
Samples in pools	53046	53046	52926	53030	52926	53030	52954	52921	52901	52901	52931	52931	52908	52931	52908	52931	52921	52954
	53085	53085	53046	53087	53046	53087	52976	53274	53023	53023	52904	52904	52904	53044	52904	53044	52930	52976
	53092	53092	52906	52907	52906	52907	53023	52969	53021	53021	53088	53088	53090	52909	53090	52909	52936	53023
	53245	53245	53048	49798	53048	49798	52919	52972	52928	52928	53093	53093	53088	49706	53088	49706	Juv-2	52919
	53251	53251	53086	52944	53086	52944	52917	52986	52941	52941	52903	52903	53273	53091	53273	53091	52940	52917
	52926	52926	53251	52922	53251	52922	52916	53020	53022	53022	53067	53067	49711	53178	49711	53178	52986	52916
	53087	53087	53245	53043	53245	53043	53022	52983	53005	53005	49706, 59898	49706, 59898	52952	53093	52952	53093	53020	53022
	52906	52906	52930	53092	52930	53092	52942	52981	53020	53020	53076	53076	53047	52905	53047	52905	52983	52942
	52907	52907	53085		53085		52978	52987	52942	52942	53273	53273	53033		53033		52981	52978
	49798	49798					52912	52974	53018	53018	52929	52929					52987	52912
	52853	52853					52913	52923	53007	53007	53047	53047					52974	52913
	53253	53253					52953	53007	52944	52944	53045	53045					52923	52953
	53048	53048					52956	52993			53044	53044					53007	52956
	52930	52930					52915	52980			53033	53033					52993	52915
	53086	53086					52914	52982			53071	53071					52980	52914
	52932	52932					Juv-1	52985			53091	53091					52982	Juv-1

	49732	49732					52957	52996			52905	52905					52985	52957
	53030	53030					52958	52995			53090	53090					52996	52958
	53043	53043					52967	52988			49711	49711					52995	52967
	29	29					52972	52971			53178	53178					52988	52930
	11	11					53274	52989			52908	52908					52971	52939
	12	12					52939	52975			53091	53091					52989	52922
	56	56					52922	52973			52909	52909					52975	52955
	53	53					52911 (cloacal)	52970			48	48					52973	52944
	52	52					52955	52991			10	10					52970	52920
	53092	53092					52911	53005			19	19					52991	52941
	52819, 53254	52819, 53254					52969	52992			52965	52965					53005	52901
	No number	No number					52944	52979			52966	52966					52992	53021
							52920	52994			52910	52910					52979	53018
							52941	52997			No number	No number					52994	52935
							52901	52984									52997	52918
							53021	52990									52984	52968
							53018	52933									52990	52940
							52935	52952									52933	Juv-2

							52918	52977									52952	52936
																	52977	
																	52968	

Supplementary Table 4. 3. Some published consensus PCR protocols used in this study.

	Herpesvirus (VanDevanter et al., 1996)		Papillomavirus (Manire et al., 2008)		Poxvirus (Li et al., 2010)	
Parameter	**Temperature (°C)**	**Duration**	**Temperature (°C)**	**Duration**	**Temperature (°C)**	**Duration**
Initial denaturation	95	15 min	95	15 min	95	15 min
Denaturation	94	30 s	94	1 min	92	10 s
Annealing	46	1 min	47	1 min	50 or 65	30 s
Extension	72	1 min	72	1 min	68	1 min
Final extension	72	7 min	72	7 min	Not applicable	Not applicable
Number of cycles	45		45		10 (followed by another 20 cycles as shown below).	
Denaturation	Not applicable		Not applicable		92	10 s
Annealing	Not applicable		Not applicable		50 or 65	30 s
Extension	Not applicable		Not applicable		68 with 2 s increment per cycle	1 min
Number of cycles	Not applicable		Not applicable		20	
PCR reagents	dNTPs (200 µM), primers (1 µM), PCR buffer (1x), DMSO (5 %), BSA (1 µg/µL) and HotStarTaq DNA polymerase (0.0025 U/µL).		dNTPs (200 µM), primers (0.75 µM), PCR buffer (1x), $MgCl_2$ (2.5 mM), and HotStarTaq DNA polymerase (0.02 U/µL).		dNTPs (350 µM), primers (20 µM), PCR buffer (1x), DMSO (2 %), Tween-20 (0.1 %), $MgCl_2$ (2.25 mM) and HotStarTaq DNA polymerase (0.04 U/µL).	
Type of PCR	Nested PCR (the same reaction conditions for the two rounds).		Conventional PCR		Conventional PCR	

Supplementary Table 4. 4. Blastn analysis of the novel viral sequences identified by consensus PCR assays.

Novel Virus	Accession number	Sequence length (bp)	Sequencing primer pair	Pool information	Most Significant Hit (Accession)	E-value	Percentage Identity
Chelid herpesvirus 1	OP076949	413	DFA and IYG	Krefft's and saw-shelled turtle oral swab from Ross River	Chelonid alphaherpesvirus 5 (MN883168.1)	1e-43	73.27
Chelid herpesvirus 2	OP076949	458	DFA and IYG	Krefft's turtle cloacal swab from Ross River	Glyptemys herpesvirus (KM357867.1)	5e-62	72.94
Chelid herpesvirus 3	OP076951	203	TGV and IYG	Saw-shelled cloacal swab from	Glyptemys herpesvirus (KM357867.1)	2e-12	70.56

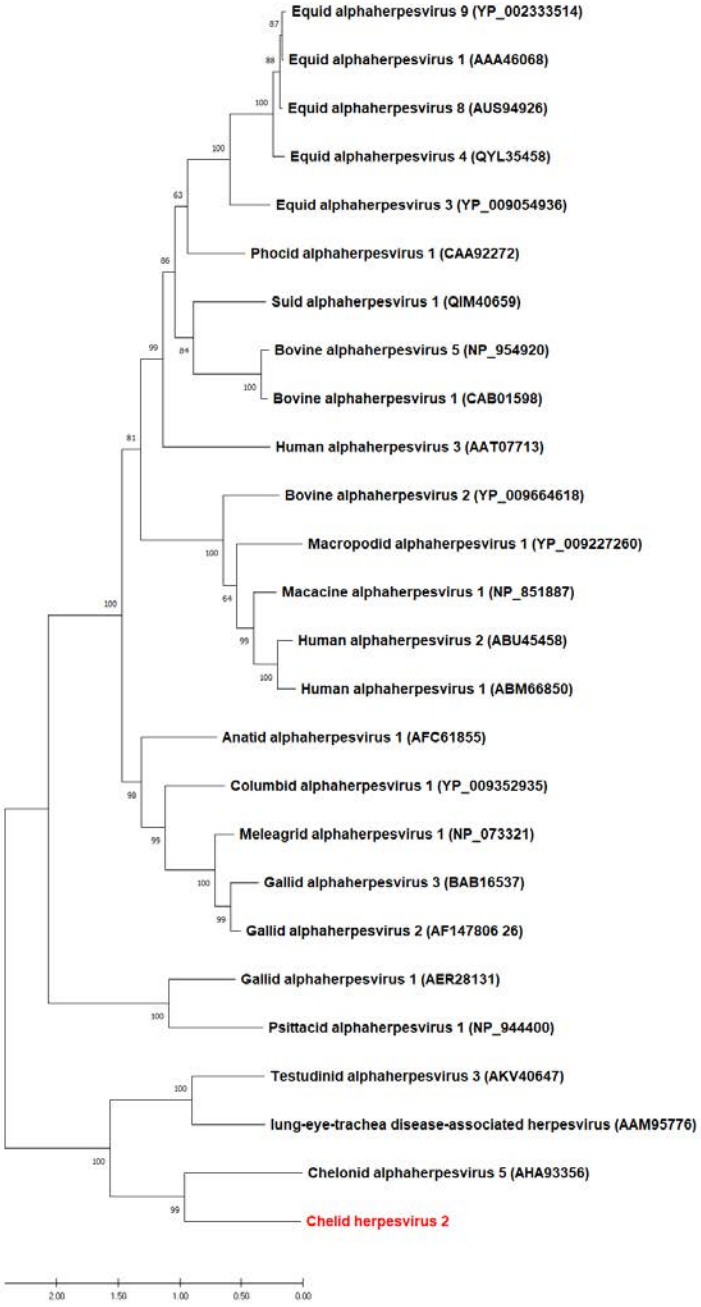
				alligator Creek			
Saw-shelled turtle adenovirus 1	OL791322	314	pol/F/inner and pol/R/inner	Saw-shelled cloacal swab from Alligator Creek	Red footed tortoise adenovirus 1 (KU601299.1)	3e-06	77.78
Saw-shelled turtle adenovirus 2	OP076952	456	pol/F/inner and pol/R/outer	Krefft's turtle cloacal swab from Ross River and Saw-shelled cloacal swab from Alligator Creek	Red-eared slider adenovirus 1 (JN632576.3)	5e-18	69.96

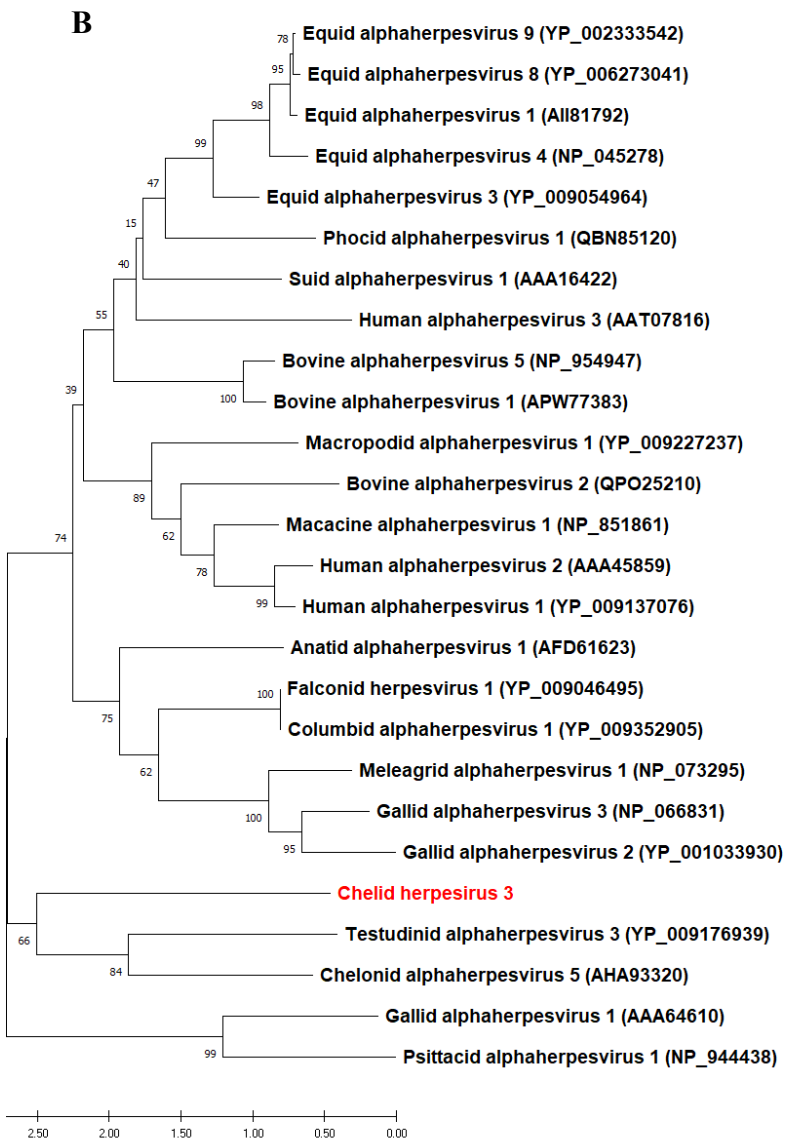
Saw-shelled turtle adenovirus 3	OP076953	259	pol/F/inner and pol/R/inner	Saw-shelled oral swab from Alligator Creek	Yellow-bellied slider adenovirus (JN632578.1)	3e-04	69.57

Supplementary Table 4. 5. Blastx analysis of sequences obtained by next generation sequencing technology.

Contig	Library	Length (bp)	Most Significant Hit (Accession)	Matched region	E-value	Percentage Identity
52955C_(paired)_contig_1497622	52955C (ChelHV-2)	675	Fibropapilloma-associated turtle herpesvirus (AAR28061.1)	Glycoprotein B	8e-63	48 %
52955C_(paired)_contig_1554826	52955C (ChelHV-2)	454	Chelonid alphaherpesvirus 5 (YP_010795529.1)	Capsid	2e-22	49%
52955C_(paired)_contig_1470460	52955C (ChelHV-2)	602	Chelonid alphaherpesvirus 5 (YP_010795561.1)	Helicase-primase subunit	1e-45	46%
52981O_(paired)_contig_1732193	52981O (ChelHV-3)	249	Chelonid alphaherpesvirus 5 (YP_010795506.1)	uracil DNA glycosylase	2e-15	54%

A


Equid alphaherpesvirus 9 (YP_002333514)
Equid alphaherpesvirus 1 (AAA46068)
Equid alphaherpesvirus 8 (AUS94926)
Equid alphaherpesvirus 4 (QYL35458)
Equid alphaherpesvirus 3 (YP_009054936)
Phocid alphaherpesvirus 1 (CAA92272)
Suid alphaherpesvirus 1 (QIM40659)
Bovine alphaherpesvirus 5 (NP_954920)
Bovine alphaherpesvirus 1 (CAB01598)
Human alphaherpesvirus 3 (AAT07713)
Bovine alphaherpesvirus 2 (YP_009664618)
Macropodid alphaherpesvirus 1 (YP_009227260)
Macacine alphaherpesvirus 1 (NP_851887)
Human alphaherpesvirus 2 (ABU45458)
Human alphaherpesvirus 1 (ABM66850)
Anatid alphaherpesvirus 1 (AFC61855)
Columbid alphaherpesvirus 1 (YP_009352935)
Meleagrid alphaherpesvirus 1 (NP_073321)
Gallid alphaherpesvirus 3 (BAB16537)
Gallid alphaherpesvirus 2 (AF147806 26)
Gallid alphaherpesvirus 1 (AER28131)
Psittacid alphaherpesvirus 1 (NP_944400)
Testudinid alphaherpesvirus 3 (AKV40647)
lung-eye-trachea disease-associated herpesvirus (AAM95776)
Chelonid alphaherpesvirus 5 (AHA93356)
Chelid herpesvirus 2
2.00
1.50
1.00
0.50
0.00



Supplementary Figure 4. 1. Midpoint-rooted Maximum Likelihood tree based on predicted amino acid sequences of herpesvirus glycoprotein B (A) uracil DNA glycosylase (B). The percentage of trees in which the associated taxa clustered together is shown next to the branches; the scale bar indicates the number of amino acid substitutions per site. The novel herpesviruses are shown in red. Evolutionary analyses were conducted in MEGA X.

Appendix Four: Supplementary Files from Chapter Five

Supplementary Table 5. 1. Relative abundance of the viral families identified by metagenomics and assigned by Krona following blastx analyses of merged sequence reads.

Sample pool	Viral family	Viral group	Read count	Relative proportion of virus (%)
Pool 2	Adenoviridae	Marsupial-associated virus	650	0.009
	Parvoviridae	Marsupial-associated virus	32	0.0004
	Picobirnaviridae	Marsupial-associated virus	1702	0.02
	Circoviridae	Marsupial-associated virus	109	0.001
	Myoviridae	Bacteriophage	400873	5
	Siphoviridae	Bacteriophage	1143340	15
	Podoviridae	Bacteriophage	1662289	22
	Herelleviridae	Bacteriophage	9194	0.1
	Ackermannviridae	Bacteriophage	1773	0.02
	Autographiviridae	Bacteriophage	243	0.003
	Schitoviridae	Bacteriophage	490	0.007
	Salasmaviridae	Bacteriophage	1382	0.02
	Demerecviridae	Bacteriophage	290	0.004
	Drexlerviridae	Bacteriophage	43	0.0006
	Microviridae	Bacteriophage	5844	0.08
	Inoviridae	Bacteriophage	3696	0.05
	Tectiviridae	Bacteriophage	320	0.004
	Tymoviridae	Plant virus	837825	11
	Virgaviridae	Plant virus	89949	1
	Geminiviridae	Plant virus	80	0.001
	Iridoviridae	Insect virus	22	0.0003
	Dicistroviridae	Insect virus	1393	0.02
	Mimiviridae	Eukaryotic virus	140	0.002
	Phycodnaviridae	Eukaryotic virus	417	0.006
	Genomoviridae	Eukaryotic virus	9	0.0001
	Marseilleviridae	Eukaryotic virus	126	0.002
	Adenoviridae	Marsupial-associated virus	1696	0.02
Pool 3	Parvoviridae	Marsupial-associated virus	1	0.00001
	Polyomaviridae	Marsupial-associated virus	1	0.00001
	Picobirnaviridae	Marsupial-associated virus	195	0.003
	Circoviridae	Marsupial-associated virus	14	0.0002
	Myoviridae	Bacteriophage	754974	11

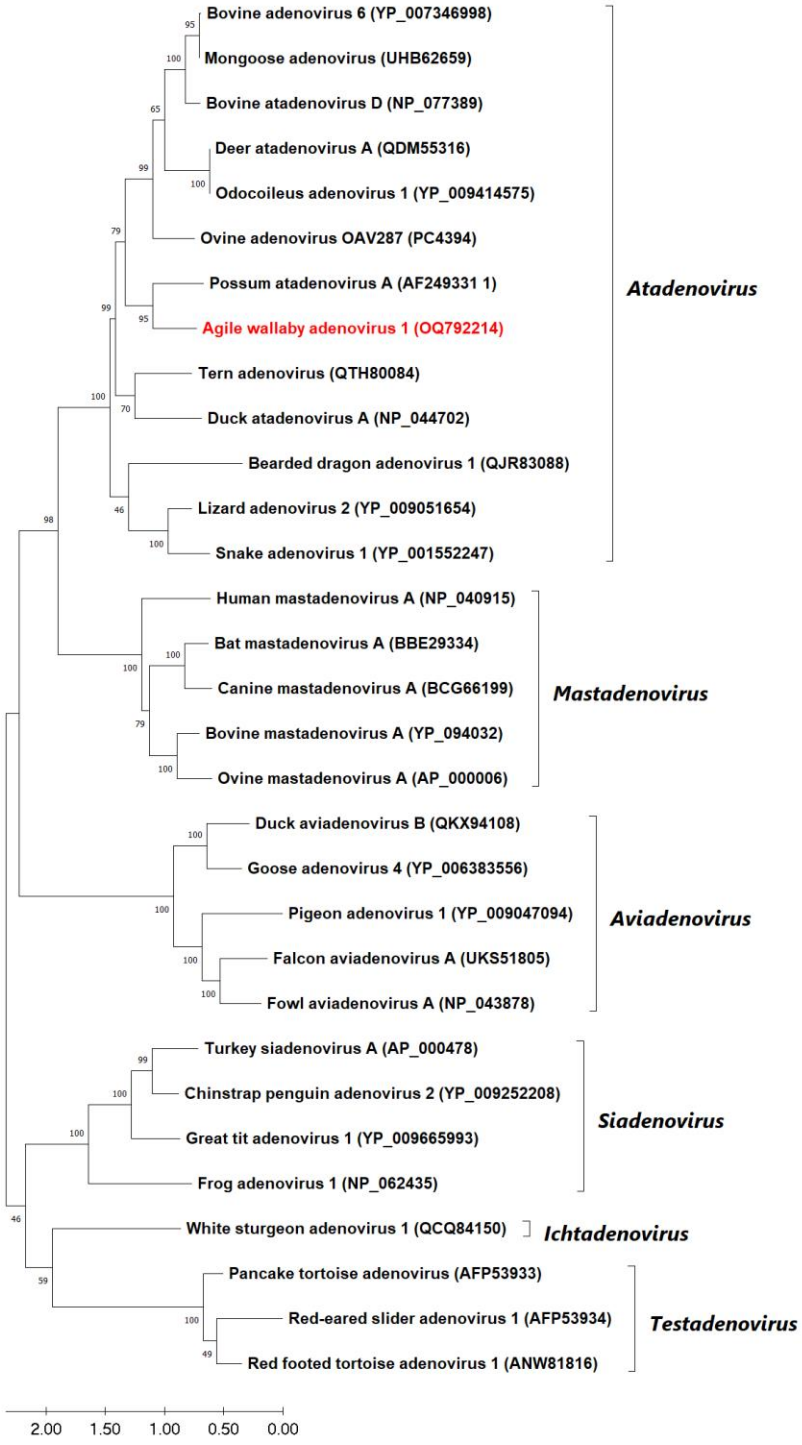
	Siphoviridae	Bacteriophage	1503356	21
	Podoviridae	Bacteriophage	1287482	18
	Herelleviridae	Bacteriophage	11707	0.2
	Ackermannviridae	Bacteriophage	2332	0.03
	Autographiviridae	Bacteriophage	240	0.003
	Schitoviridae	Bacteriophage	719	0.01
	Salasmaviridae	Bacteriophage	371	0.005
	Demerecviridae	Bacteriophage	355	0.005
	Drexlerviridae	Bacteriophage	52	0.0007
	Microviridae	Bacteriophage	3409	0.05
	Inoviridae	Bacteriophage	914	0.01
	Tectiviridae	Bacteriophage	139	0.002
	Tymoviridae	Plant virus	87723	1
	Virgaviridae	Plant virus	17291	0.2
	Geminiviridae	Plant virus	53	0.0007
	Iridoviridae	Insect virus	12	0.0002
	Dicistroviridae	Insect virus	244	0.003
	Poxviridae	Insect virus	1	0.00001
	Mimiviridae	Eukaryotic virus	137	0.002
	Phycodnaviridae	Eukaryotic virus	858	0.01
	Genomoviridae	Eukaryotic virus	3	0.00004
	Marseilleviridae	Eukaryotic virus	281	0.004

Supplementary Table 5. 2. List of marsupial-associated viruses identified by metagenomics and PCR, including information on the contig name, coverage depth, library, classification, virus name, GenBank accession number and sequence length.

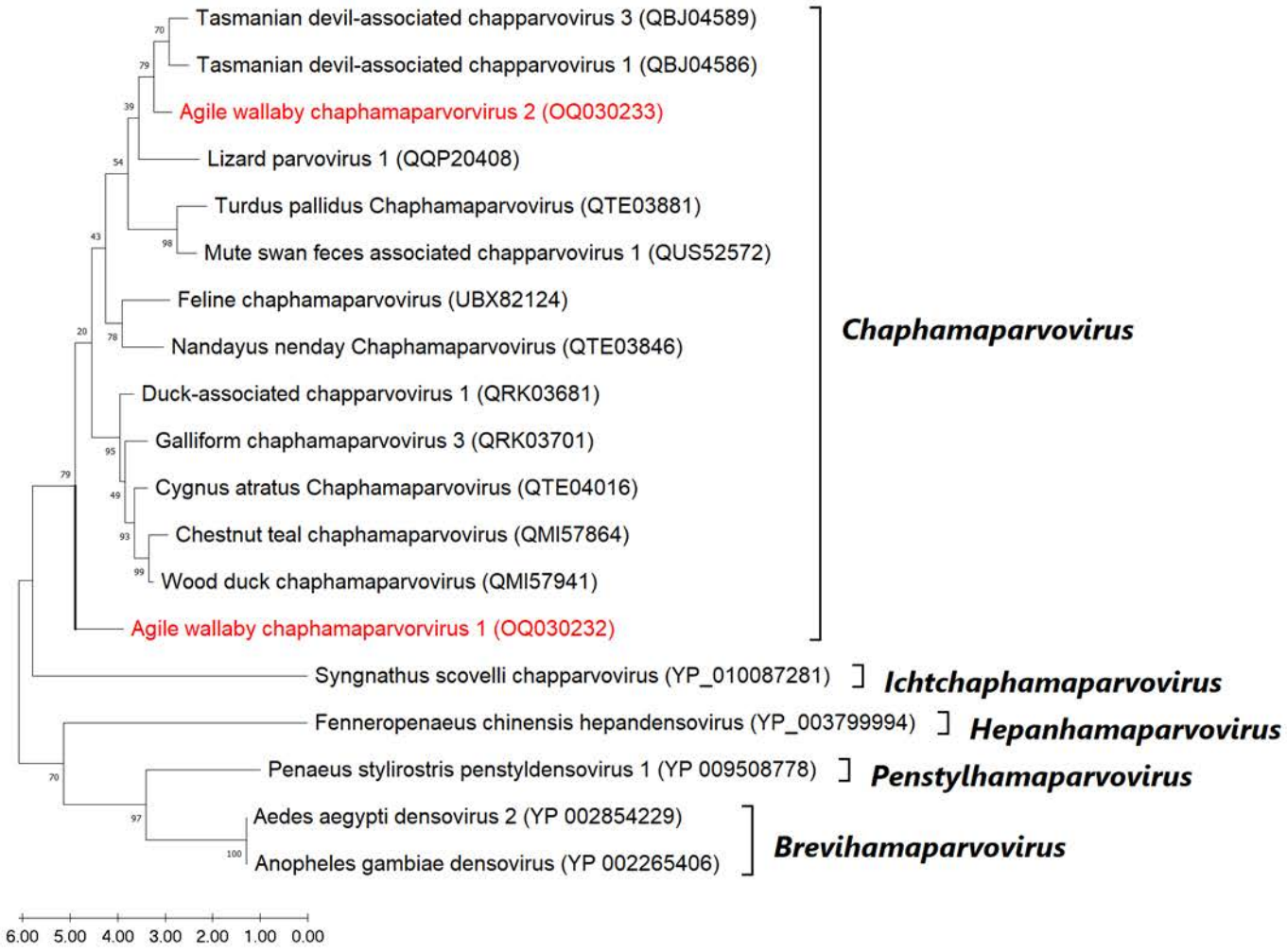
Contigs name	Library	Classification	Virus name	GenBank accession number	Mean coverage depth*	Breadth of coverage (%)*	Original length (before editing)	Length (after editing)
NODE_528_length_31261_cov_13.970583	GOP3	Adenoviridae	Agile wallaby atadenovirus 1	OQ030229	22x	100	2730	422
NODE_154279_length_308_cov_5.569170	GOP2	Parvoviridae	Agile wallaby chaphamaparvovirus 1	OQ030230	7x	92	308	308
NODE_311983_length_230_cov_1.325714	GOP2	Parvoviridae	Agile wallaby chaphamaparvovirus 2	OQ030231	2x	100	230	230
NODE_240878_length_246_cov_1.005236	GOP3	Polyomaviridae	Agile wallaby Polyomavirus 1	OQ030232	1x	100	246	246
WR9_contig	GOP3	Polyomaviridae	Agile wallaby Polyomavirus 2	OQ030233	Not applicable	Not applicable	293	223
NODE_16197_length_1712_cov_38.624019	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 1	OQ030234	65x	100	1712	349
NODE_19056_length_1508_cov_9.660702	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 2	OQ030235	15x	100	1508	267
NODE_260071_length_249_cov_1.484536	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 3	OQ030236	2x	100	249	249
NODE_197756_length_275_cov_2.618182	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 4	OQ030237	3x	100	275	275

NODE_25504_length_1194_cov_5.513608	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 5	OQ030238	8x	100	1194	444
NODE_26346_length_1162_cov_10.381210	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 6	OQ030239	16x	100	1162	284
NODE_34127_length_950_cov_7.189944	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 7	OQ030240	10x	92	950	320
NODE_39317_length_852_cov_4.154329	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 8	OQ030241	7x	100	852	666
NODE_86759_length_454_cov_2.162907	GOP2	Picobirnaviridae	Agile wallaby-associated picobirnavirus 9	OQ030242	3x	100	454	356
NODE_272725_length_244_cov_2.031746	GOP2	Circoviridae	Circovirus	OQ030243	2x	100	244	244
WR13_contig	GOP2	Circoviridae	Cyclovirus	OQ030244	Not applicable	Not applicable	478	360
WR22	GOP2	Herpesviridae	Macropod gammaherpesvirus 3	Not accessioned	Not applicable	Not applicable	223	153

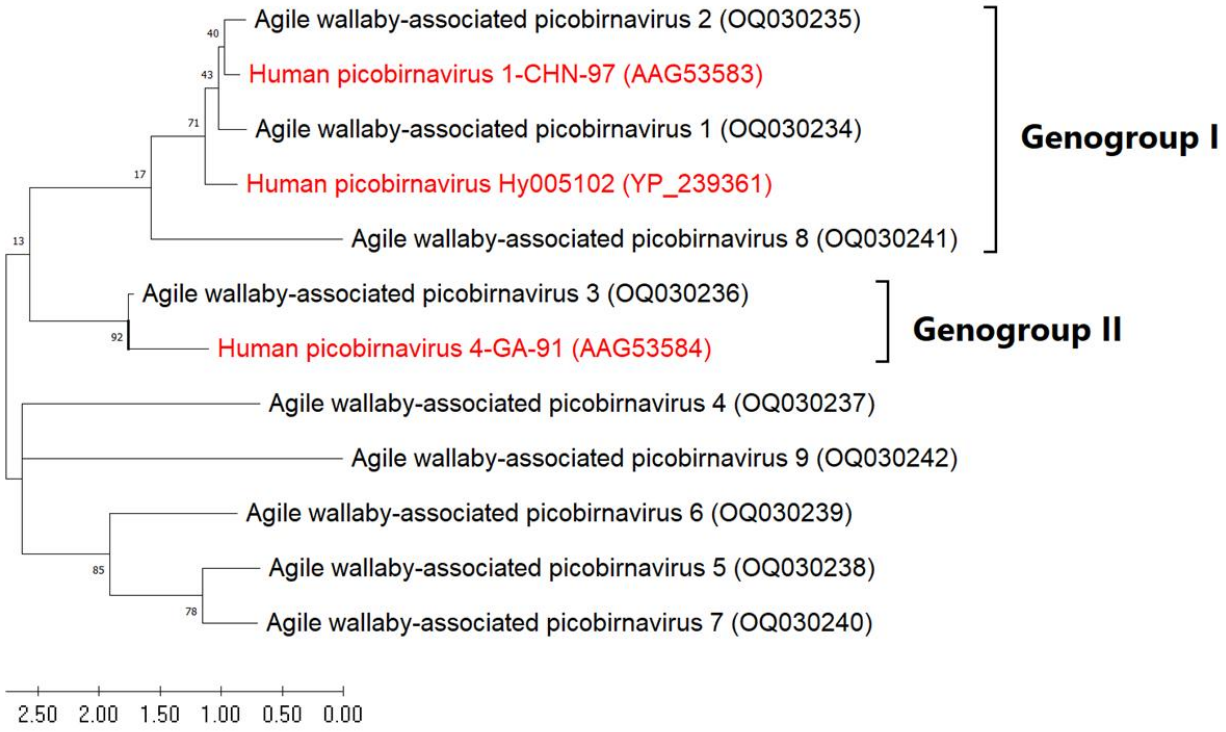
*mean depth and breadth of coverage were calculated using SAMtools (Danecek et al., 2021) following read alignment to contigs with Bowtie 2 (Langmead & Salzberg, 2012).



Supplementary Figure 5. 1. Genus demarcation of agile wallaby atadenovirus 1 (red ink) based on the amino acid sequences of DNA polymerase. Numbers at nodes indicated level of bootstrap support calculated from 1000 replicates; the scale bar indicates the number of amino acid substitutions per site.



Supplementary Figure 5. 2. Genus demarcation of agile wallaby chaphamaparvirus 1 and 2 (red ink) based on the amino acid sequences of NS1. Numbers at nodes indicated level of bootstrap support calculated from 1000 replicates; the scale bar indicates the number of amino acid substitutions per site.



Supplementary Figure 5. 3. Phylogenetic analysis of the RdRp of agile wallaby-associated picobirnaviruses and their relationship with the reference sequences of genogroup I and II (shown in red). Numbers at nodes indicated level of bootstrap support calculated from 1000 replicates; the scale bar indicates the number of amino acid substitutions per site.

Appendix Five: Publications arising during candidature

In this appendix, the following published articles are provided:

Okoh, G.R., Horwood, P.F., Whitmore, D. and Ariel, E. (2021). Herpesviruses in Reptiles. *Frontiers in Veterinary Science*, 8:642894. Doi: 10.3389/fvets.2021.642894

Okoh, G.R., Lockhart, M., Grimsey, J., Whitmore, D., Ariel E., Butler, J. and Horwood P.F. (2023). Development of Subfamily-Based Consensus PCR Assays for the Detection of Human and Animal Herpesviruses. *European Journal of Clinical Microbiology and Infectious Diseases*. Doi: 10.1007/s10096-023-04605-w

Okoh, G.R., Ariel, E., Whitmore, D. and Horwood, P.F. (2023). Draft Genome Sequence of a Novel Adenovirus Recovered from the Metagenome of Agile Wallabies. *Microbiology Resource Announcements*, e00112-23. Doi: 10.1128/mra.00112-23

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REVIEW
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Herpesviruses in Reptiles

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Since the 1970s, several species of herpesviruses have been identified and associated with significant diseases in reptiles. Earlier discoveries placed these viruses into different taxonomic groups on the basis of morphological and biological characteristics, while advancements in molecular methods have led to more recent descriptions of novel reptilian herpesviruses, as well as providing insight into the phylogenetic relationship of these viruses. Herpesvirus infections in reptiles are often characterised by non-pathognomonic signs including stomatitis, encephalitis, conjunctivitis, hepatitis and proliferative lesions. With the exception of fibropapillomatosis in marine turtles, the absence of specific clinical signs has fostered misdiagnosis and underreporting of the actual disease burden in reptilian populations and hampered potential investigations that could lead to the effective control of these diseases. In addition, complex life histories, sampling bias and poor monitoring systems have limited the assessment of the impact of herpesvirus infections in wild populations and captive collections. Here we review the current published knowledge of the taxonomy, pathogenesis, pathology and epidemiology of reptilian herpesviruses.

Keywords: herpesviruses, reptiles, fibropapillomatosis, taxonomy, pathogenesis, pathology, epidemiology

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INTRODUCTION

Reptiles are a group of vertebrates (class Reptilia) that are adapted to a broad range of terrestrial and aquatic environments (1, 2). The group comprises over 11,000 extant species placed in four orders, namely: Testudines (turtles, tortoises, and terrapins); Squamata (lizards, snakes, and worm lizards); Crocodilia (crocodiles, alligators, gharials, and caimans); and Rhynchocephalia (tuatara) (3). Reptiles constitute an integral part of the natural ecosystem and play the roles of both pollinators and predators, as well as environmental health indicators (4). In addition to their ecological services, reptiles have become desirable for food, medicinal products, pet trade, leather goods and research applications (5–7). However, their existence and well-being have constantly been threatened by several factors, such as hunting, environmental pollution, loss of habitat, destructive non-native species, climate change, and infectious diseases (4, 8–10). Disease surveillance and research in wild populations of reptiles are associated with numerous challenges including difficulties in accessing samples or field data, misleading epidemiological data and missing population data, as well as political and cultural restrictions (11). These challenges well explain the use of captive wildlife as models in many studies to acquire epidemiological information, since diseases are comparable in both wild and captive animals (12–15). Nonetheless, more robust and ideal epidemiological data are obtained when free ranging animals are surveyed. Recently, researchers have taken a renewed interest in reptilian viruses, partly due to the role played by reptiles as reservoir hosts for zoonotic viruses, as well as improvements in viral diagnostic methods that, in turn, have increased understanding of viruses in reptiles (16–20).

Herpesviruses (HVs) are members of the family *Herpesviridae,* a large taxon of DNA viruses that have been described in most vertebrate animals, including reptiles (16, 21). Herpesviruses are enveloped viruses with an icosahedral nucleocapsid and a linear double-stranded genome of varying length from ∼124 to 259 kbp (22). Generally, HVs replicate within host cell nuclei and are able to remain latent in their natural hosts (17, 23). So far, reptilian HVs that have been identified and characterised all belong to the subfamily *Alphaherpesvirinae* (17, 24–26).

The occurrence of HV infections among reptiles has been widely documented and associated with stomatitis, tumors, encephalitis, conjunctivitis, hepatitis and mortalities (27, 28). Unfortunately, current treatment options of reptilian HVs are limited and the search for potent vaccines remains a herculean task; therefore, the adoption of preventative strategies is still the most efficient way of controlling these diseases. This review aims to assist in biosecurity planning as well as create a knowledge platform for decision makers and researchers by providing an overview of the taxonomy, pathogenesis, pathology and epidemiology of reptilian HVs.

METHODS

Databases such as Medline (Ovid), PubMed, and Scopus were searched using specific keywords and phrases including *Herpesviridae* infections, herpesvirus infection, fibropapillomatosis, grey-patch disease, loggerhead genital-respiratory herpesvirus, herpesvirus disease, reptiles, turtles, tortoise, snakes, lizards, alligators, and crocodiles (**Supplementary File 1**). To ensure that relevant publications were not missed, each subheading was searched independently on PubMed. Also, an additional literature search was conducted by assessing references of articles selected from previous databases. A summary of the search results is shown in **Figure 1**. Furthermore, we read the abstracts and full texts of the selected articles, extracted and analysed information on the diagnostic methods used and the reptilian HVs investigated from 1972 to September 8, 2020 (**Figures 2**, **3**; **Supplementary File 2**). Non-English, non-original research, guidelines, and review articles were excluded from the analysis.

BIBLIOMETRICS

We conducted bibliometric analyses of published articles on the topic of reptilian HVs using Vosviewer software (29) and the Web of Science Core Collection database. A total of 245 publications were downloaded from Web of Science Core Collection database using the following search terms; herpesvirus, turtle, lizard, snake, tortoise and crocodile. The strategy involved a combined use of the keywords, tags and Boolean operators to create query sets as follows: ALL= (herpesvirus) AND ALL= (turtle* OR lizard* OR snake* OR tortoise* OR crocodile*) with no limitations. USA had the highest number of research outputs with 149 (60.8%) articles. This was followed by Germany (28; 11.4%) and Australia (26; 10.6%) (**Figure 4**; **Table 1**). Using Vosviewer, we visualised the major keywords commonly used in the field of reptilian HVs and the link strengths between collaborating countries (**Figure 4**; **Supplementary File 3**). Of note, a low number of records on reptilian HVs were observed for some countries (Indonesia, Mexico and India) that have rich reptile diversity (**Table 1**) (3, 30, 31). Some of these countries also had little or no collaborations with the high research output countries (**Figure 4**), thus suggesting an under-reporting of reptilian HVs in these countries. Conversely, Germany has less reptile diversity with a higher number of records (**Table 1**). This observation could be attributed to the presence of established diagnostic resources or increased monitoring and reporting systems for reptilian diseases in the country.

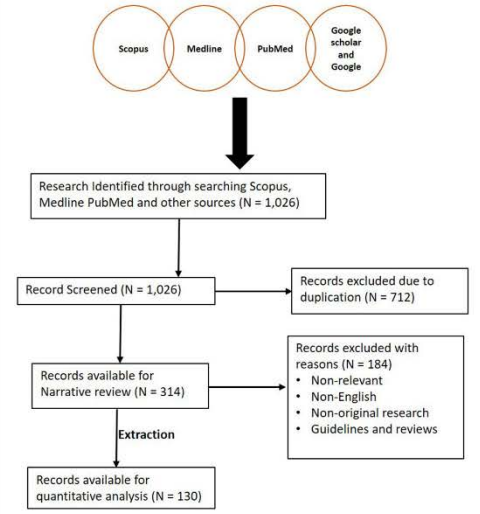

FIGURE 1 | Summary of literature search conducted. A total of 1,026 articles were initially screened and 712 articles were later excluded due to duplication. A total of 314 articles were then reviewed for this study. One hundred and thirty studies were then extracted for quantitative analysis.

TAXONOMY OF REPTILIAN HERPESVIRUSES

Reptilian HVs belong to the family *Herpesviridae,* a member of the order *Herpesvirales* (32). According to the 2019 *International Committee on Taxonomy of Viruses* (ICTV) classification, the subfamily *Alphaherpesvirinae* comprises five genera namely, *Iltovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus,* and *Varicellovirus*. Only the genus *Scutavirus* contains species that cause HV diseases in reptiles and includes *Chelonid alphaherpesvirus 5* (ChHV-5) and *Testudinid alphaherpesvirus*

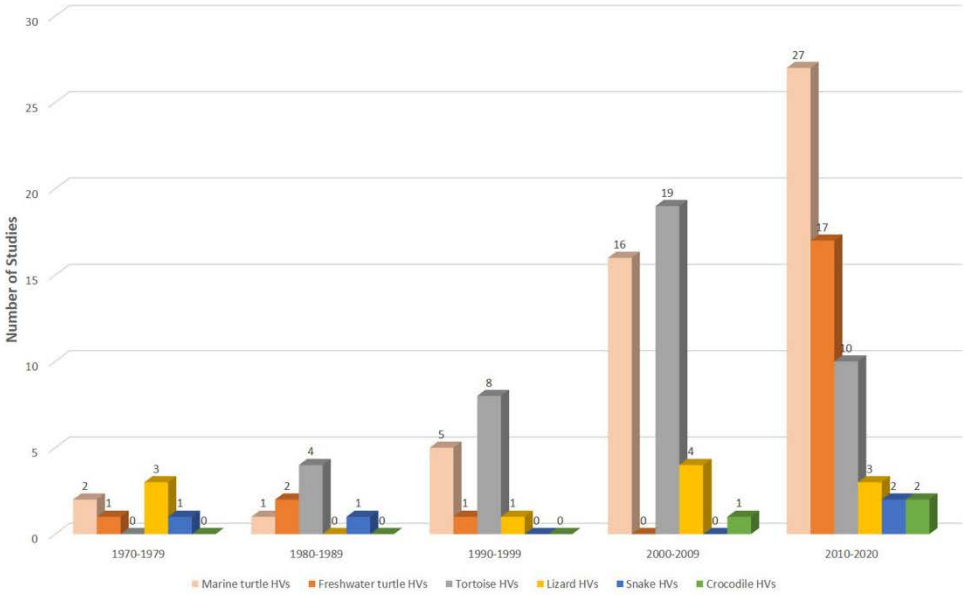

FIGURE 2 | Studies on HVs in reptiles from 1972 to September 8, 2020. Overall, more than a quarter ($n = 51$; 39%) of the 130 extracted studies were conducted in marine turtles since the 1970s. Of the 60 publications between 2010 and 2020, 45% ($n = 27$) were ChHV-5 related studies.

3 (TeHV-3). The species *Chelonid alphaherpesvirus 6* (ChHV-6) belongs to the subfamily *Alphaherpesvirinae* with unknown generic placement. The *Iguanid herpesvirus 2* (IgHV-2) that causes cytopathic infection in iguanids is of unknown generic and subfamilial placement (32–35).

Since the end of the twentieth century, advances in molecular and phylogenetic analyses have made it possible for novel reptilian HVs to be identified with proposed taxonomic placements (36). Novel HVs have been detected in freshwater turtles, including Emydoidea herpesvirus 1 (EBHV-1), Pelomedusid herpesvirus 1, Glyptemys herpesvirus 1 and 2 (GlyHV-1 and−2), Emydid herpesvirus 1 and 2 (EmyHV-1 and−2), and Terrapene herpesvirus 1 and 2 (TerHV-1 and−2) (28, 37–41). Loggerhead genital-respiratory herpesvirus (LGRV) and loggerhead orocutaneous herpesvirus (LOCV) were detected in loggerhead turtles (*Caretta caretta*) and the genus *Chelonivirus* was proposed for these viruses and other related chelonian HVs (42). Also, tortoise HV species (TeHV-1,−2,−3,−4) have been identified and placed in the proposed genus Chelonivirus (43–46), although TeHV-3 has been formally assigned to the genus *Scutavirus* (32). Other unassigned reptilian HVs in the family *Herpesviridae* include the Iguanid herpesvirus 1 (IgHV-1), Gerrhosaurid herpesvirus 1-3, Varanid herpesvirus (VHV-1,−2,−3) and Helodermatid herpesvirus 1 (HeHV-1) in lizard species (25, 47–51), Opheodrys herpesvirus 1 in snakes (52), Crocodyline herpesvirus 1-3 (CrHV-1,−2,−3) in crocodiles (53), and, Chelonid herpesvirus 1-4 (ChHV-1,−2,−3,−4) in green turtles (*Chelonia mydas*-ChHV-1) (54), freshwater turtles (*Clemmys marmorata*-ChHV-2; *Chrysemis picta*-ChHV-3) (55, 56), and Argentine tortoise (*Geochelone chilensis*-ChHV-4) (57). Notably, some of these unassigned HVs were identified decades ago based on their morphological and biological characteristics using techniques (virus isolation, electron microscopy and histopathology) that were available at that time, thus making it challenging to place them taxonomically. We conducted a phylogenetic analysis to illustrate the relationship between the unassigned reptilian HVs and currently assigned HVs using amino acid sequences of HV-DNA-dependent DNA polymerase (37 complete and 17 partial sequences) from the NCBI website (https://www.ncbi.nlm.nih.gov/). As previously described (37, 58), the analysis showed that the unassigned reptilian HVs form a monophyletic group with members of the subfamily *Alphaherpesvirinae*. Freshwater HVs showed a close phylogenetic relationship with the tortoise HVs while the lizard HVs indicated high variations (**Figure 5**). Overall, given the variations shown by the unassigned reptilian HVs, it remains a matter of scientific deliberation whether these viruses should be assigned into one genus such as TeHV-3 and ChHV-5 or into different genera, although we envisage the latter would be the case.

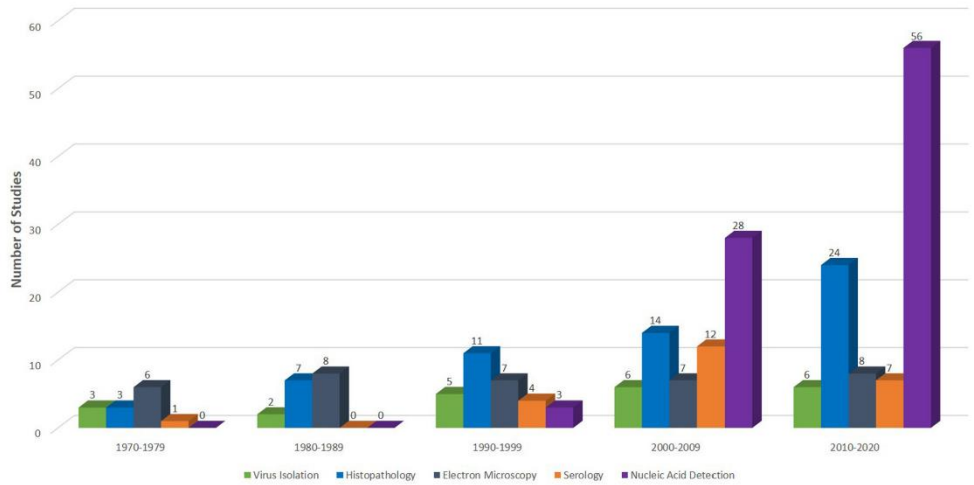

FIGURE 3 | Diagnostic methods used in different studies from inception 1970 to September 8, 2020. Of the 130 studies extracted for this quantitative analysis, 22 (17%), 59 (45%), 36 (28%), 24 (18%), and 87 (67%) used virus isolation, histopathology, electron microscopy, serology, and nucleic acid detection assays, respectively, for various investigations of reptilian HVs.

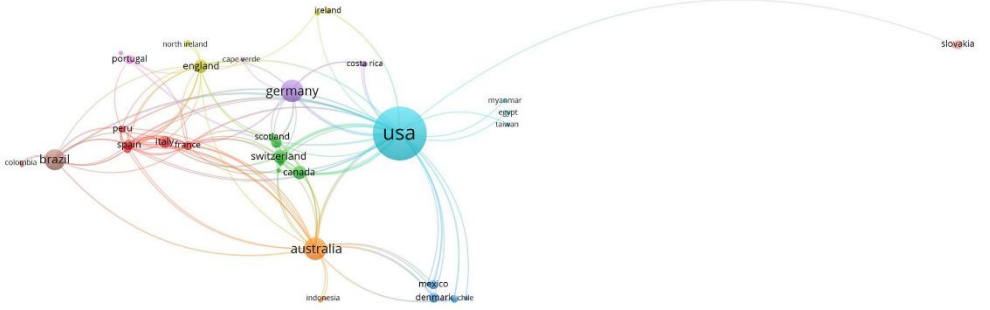

FIGURE 4 | Links between collaborating authors from different countries with research output from inception to 2020. The circular coloured nodes represent countries and the node size indicates number of publications from the country. The lines between nodes indicate authorship collaborations between countries and the widths of these lines indicate the link strength.

VIRION AND GENOME ORGANIZATION

All members of the family *Herpesviridae* share a common virion architecture, comprising a monopartite, linear, double stranded DNA core enclosed within an icosahedral capsid with a $T = 16$ symmetry (60–62). The capsid is tightly wrapped by a proteinaceous tegument, which, in turn, is surrounded by an envelope containing polyamines, lipids, and essential antigenic glycoproteins (62, 63). Unlike reptilian HVs, the atomic structures of human alphaherpesviruses have mostly been described. For instance, a cryo-electron microscopy (Cryo-EM) resolved the atomic structure of human simplexviruses (HSV-1 and−2), which comprise capsid organisation (hexons, pentons and triplexes), capsid proteins (VP5, VP19C, VP23, and VP26) and tegument proteins (pUL17, pUL25, and pUL36) (64–66). Although the atomic structures of reptilian HVs have not specifically been resolved, the resolved structures of other alphaherpesviruses provide insights, since their genomes have many similarities (36, 67). Consequently, the insights could guide future

TABLE 1 | Bibliographic data on reptilian herpesviruses based on the number of articles from different countries.

Countries/Regions	Records	% of 245	No. of reptiles species (3,31)	*Rank
USA	149	60.8	1,147	2
Germany	28	11.4	29	31
Australia	26	10.6	1,159	1
Brazil	23	9.4	878	5
Switzerland	11	4.5	27	32
Italy	10	4.1	65	24
Canada	9	3.7	57	25
United Kingdom	15	6.1	6	43
Belgium	6	2.4	11	41
Spain	6	2.4	78	23
Denmark	5	2.0	12	40
France	4	1.6	181	15
Mexico	4	1.6	1,021	3
Portugal	4	1.6	44	27
Slovakia	4	1.6	16	37
Ecuador	3	1.2	541	10
Japan	3	1.2	111	22
China	3	1.2	605	8
Peru	3	1.2	586	9
Austria	2	0.8	18	36
Costa Rica	2	0.8	467	12
Czech Republic	2	0.8	15	38
India	2	0.8	889	4
Ireland	2	0.8	21	35
Netherlands	2	0.8	23	34
Barbados	1	0.4	13	39
Cape Verde	1	0.4	51	26
Chile	1	0.4	179	16
Colombia	1	0.4	654	7
Croatia	1	0.4	42	28
Egypt	1	0.4	133	20
French Guiana	1	0.4	177	17
Indonesia	1	0.4	798	6
Israel	1	0.4	148	19
Myanmar	1	0.4	374	13
Nicaragua	1	0.4	218	14
Norway	1	0.4	10	42
Romania	1	0.4	31	30
Seychelles	1	0.4	37	29
South Africa	1	0.4	529	11
South Korea	1	0.4	26	33
Taiwan	1	0.4	123	21
Turkey	1	0.4	150	18
Turks Caicos	1	0.4	12	40

**Ranking was conducted based on the number of reptile species by countries identified from our bibliometric search and not based on the global ranking by Butler (31).*

research in the atomic structure resolutions of reptilian HVs, which in turn could serve as a baseline for reptilian HV vaccinology.

Although the complete nucleotide sequences for most reptilian HVs are yet to be obtained, genomic features can be inferred from other fully sequenced alphaherpesviruses owing to sequence homology (36, 67). All alphaherpesvirus genome structures contain unique long (U_L) and short (U_S) sequences and each are flanked by both terminal (TR_L, TR_S) and internal (IR_L, IR_S,) inverted repeat regions, giving the general configuration TR_L-U_L-IR_L-IR_S-U_S-TR_S (68). The complete genome of two TeHV-3 strains (1976 and 4295) was recently sequenced. The 1976 strain was shown to have a novel inverted repeat (TR_T, IR_T) and unique (U_T) regions (69). The genome is approximately 160 kbp, encodes at least 107 open reading frames (ORFs) and consists of U_L (107,928 bp) and U_S (20,375 bp) regions. The U_L is bound to its right by the U_S adjoined to inverted repeats (IR_S and TR_S; 8,536 bp) and to its left by a third unique region (U_T; 12,595 bp), which is also bordered by inverted repeats (TR_T and IR_T; 1,194 bp) to give the overall layout TR_T-U_T-IR_T-U_L-IR_S-U_S-TR_S (69). However, this differs from the type D configuration earlier attributed to this species (70). In another study, the complete nucleotide sequence of a Bacterial Artificial Chromosome (BAC) containing the entire genome of ChHV-5 (cloned in pTARBAC2.1) was obtained and it showed a different configuration (U_L-IR_S-U_S-TR_S) from that of TeHV-3, even though they both belong to the genus *Scutavirus* (71). Moreover, the genome characterisation of strain 4295 identified regions containing genes that could be involved in viral pathogenesis or virulence (69). This is an important finding as these regions could serve as therapeutic or diagnostic targets in future research. Similarly, evidence of recombination among strains of ChHV-5 documented by Morrison et al. could lead to increased virulence and transmission of ChHV-5 variants (72) and these events may remain undetected in sea turtle populations. Therefore, it is pertinent to strengthen current diagnostic approaches to allow for more comprehensive geographical surveys and characterisation of HVs. Also, as new and affordable diagnostic techniques are being developed and improved upon, we expect more novel structures of reptilian HVs to be reported.

TRANSMISSION AND PATHOGENESIS

Several modes of reptilian HV transmission have been postulated including vertical, horizontal and mechanical transmissions (73–75). Marenzoni et al. reported the first evidence of vertical transmission of TeHV-3 in a captive breeding facility (14). In this study, one hatchling born in isolation from the egg laid by an infected tortoise (*Testudo hermanni hermanni*) presented with conjunctivitis and tested positive by specific polymerase chain reaction (PCR) targeting the partial sequence of the UL39 gene of TeHV-3 (14). In other studies, Jones and colleagues provided molecular evidence for the horizontal transmission of ChHV-5 in green turtles by demonstrating the molecular link between viral variants and foraging grounds (76, 77). Furthermore, experimental studies have revealed the possible transmission of reptilian HVs by direct contact with infectious

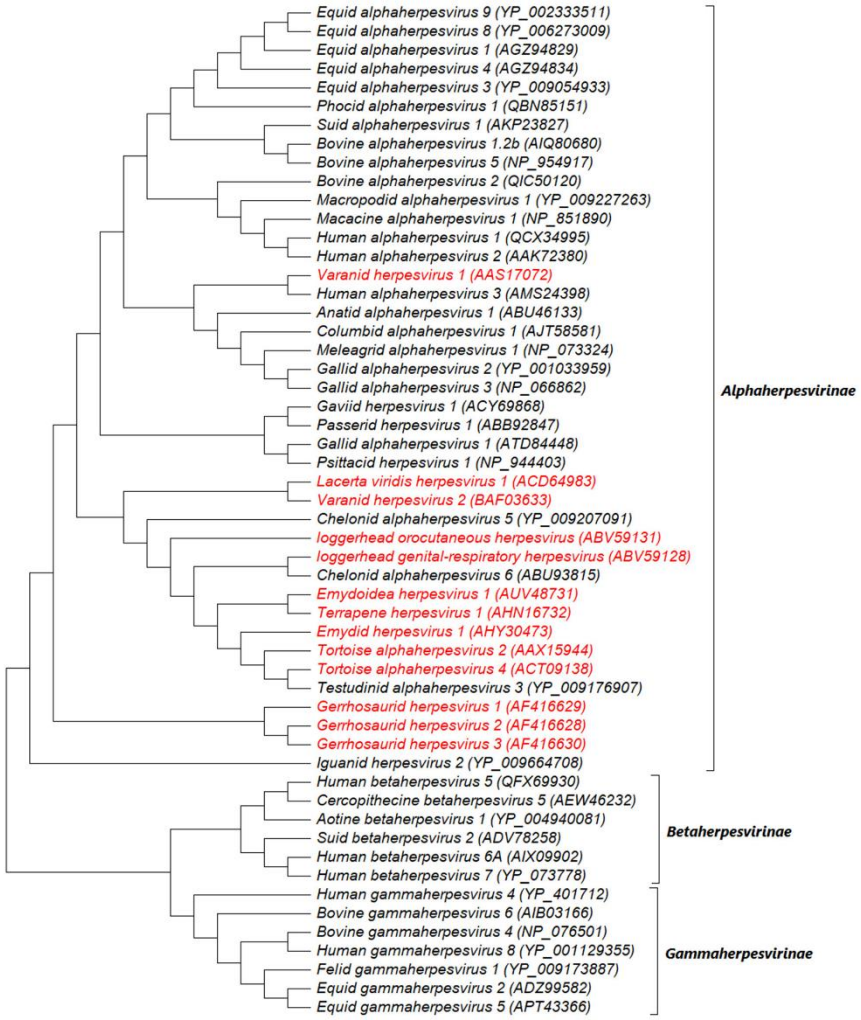

FIGURE 5 | Midpoint rooted maximum likelihood phylogenetic tree of predicted amino acid sequences of HV-DNA-dependent DNA polymerase. The unassigned reptilian HVs are shown in red and cluster within the subfamily *Alphaherpesvirinae*. This analysis involved 54 amino acid sequences (37 complete and 17 partial sequences) downloaded from NCBI website (https://www.ncbi.nlm.nih.gov/) and aligned by ClustalW. There were a total of 1,443 positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The tree with the highest log likelihood (−60139.09) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (59).

secretions (78–80) or indirectly via vectors and water (81–84). By linking viral shedding patterns or frequency to disease occurrence, we could trace the most probable transmission mode of reptilian HVs. For instance, in captive enclosures, HVs could be easily transmitted via contact with secretions or contaminated materials, even at low shedding rates, and

the removal of infected animals and infectious materials could stop the spread of the virus. In the wild, different transmission agents such as vectors, fomites and superspreaders could interplay to compensate for the low contact rate and infrequent shedding of some HVs (85). Because of the managerial implications, it is therefore important to further investigate the roles of these factors in the transmission of HVs in wild reptiles.

Four pathogenic mechanisms are highly conserved among all HVs and include: (1) intranuclear replication and capsid assembly; (2) expression of DNA metabolic and synthetic enzymes; (3) cell destruction following the release of viral progenies; and (4) the maintenance of latency in natural host cells (63, 86, 87). Generally, HV infections begin with viral entry, which is followed either by localisation or systemic spread (87). An experimental transmission study described the systemic dissemination of tortoise HVs (isolates HV 1976 and HV 4295/7R/95) (78). Following experimental infection via intranasal and intramuscular routes, the HVs elicited clinical signs and were detected by PCR in tissue samples from the respiratory, digestive and urogenital tracts, central nervous system (CNS), heart and spleen (78). Fibropapillomatosis (FP) and grey patch disease (GPD) are both associated with clinical signs that could be attributed to local destruction of infected cells due to replication and progeny release (54, 80, 88–90). Evidentially, two studies demonstrated the local replication of ChHV-5 by detecting certain biomarkers (eosinophilic intranuclear inclusions, F-VP26, DNA, and mRNA transcripts) within fibropapillomatous lesions (85, 91).

Unfortunately, the specific mechanisms involved in host cell invasion, immune evasion, localisation and spread of reptilian HVs have not been fully elucidated. However, recent molecular studies have provided insights into some virulence factors associated with reptilian HVs (69–71). Briefly, glycoproteins B (gB), gC, gD, gH, gK gL, gM, and gN have been hypothesised to function in host cell attachment and entry (69–71). gB and gC are capable of binding to heparan sulphate proteoglycans that are present on the surface of many cells, thus aiding viral adsorption and penetration into different cell types (69, 92). The interactions of gB, gD, gH, gK, gL, gM, and gN have also been postulated to mediate membrane fusion and viral entry into the cell (69, 70, 92). Glycoproteins B, E, H, and L are involved in viral cell to cell spread, which could occur through intercellular bridges or intra-axonal transport, thus circumventing humoral immune responses (69, 70). Similarly, the gC can bind to the third complement component (C3b) to block the alternative pathway complement activation (69, 93). The gE and gI in HSV-1 inhibit the normal function of antibodies by building up a complex that acts as an Fc-receptor (94). However, the immunosuppression mechanism of the gE homologue in reptilian HVs is not yet clear. Finally, the F-M04 and F-sial proteins were recently identified in ChHV-5 and thought to play a role in FP pathogenesis; however, the specific mechanisms involved are not yet understood (71). Future research should consolidate characterisation of reptilian HVs in order to increase the understanding of host-pathogen interactions and improve clinical interventions.

CLINICAL AND PATHOLOGICAL SIGNS

Herpesvirus infections have been described in reptiles with a range of clinical manifestations (16). To provide an overview, the clinical signs and the gross and histological lesions associated with reptilian HVs are summarised in **Table 2**. Some of the more detailed descriptions are from sea turtles, tortoises and crocodiles. Grey patch disease and FP, characterised by coalescing greyish papular skin lesions (spreading patches) and branching papillary tumours (**Figure 6**), respectively, have been reported in sea turtles (42, 54, 108, 109). Lung-eye-trachea disease (LETD) with a clinical course of 2–3 weeks has been seen in green sea turtles (98). Lung-eye-trachea diseased turtles often present with pneumonia, stridor and caseation of the eyes, oropharynx and trachea (98). In freshwater turtles, HV infections are associated with hepatic necrosis, and proliferative and/or ulcerative lesions of the skin and shell (**Figure 7**) (37, 38, 110, 111). Infections in tortoises result in ulcerative to diphtheroid-necrotizing stomatitis, conjunctivitis, glossitis, rhinitis, dyspnoea, liver disease and neurological disease and could be accompanied by anorexia, regurgitation, neck oedema, lethargy and death (**Figure 8**) (112–115). Papillomas, stomatitis, and hepatitis are commonly described in lizards infected with HV (25, 47–49, 116, 117). Recently, five green snakes (*Opheodrys vernalis*) housed together presented with oropharyngeal squamous cell carcinoma and molecular analysis confirmed the presence of a novel Opheodrys herpesvirus-1 (*Alphaherpesvirinae*) (52). In another study, a lymphoid follicular cloacal inflammation in juvenile alligators was initially associated with tortoise HV. However, the HV (Genbank accession AY913769.1) was later determined to be a likely laboratory contamination and the actual causative agent is still unknown (118). Similarly, Hyndman et al. identified three novel HVs associated with conjunctivitis and/or pharyngitis (CP), systemic lymphoid proliferation with non-suppurative encephalitis (SLPE), and lymphonodular skin lesions (LNS) in farmed saltwater crocodiles (*Crocodylus porosus*) and captive freshwater crocodiles (*Crocodylus johnstoni*) (53). Obviously, HVs can induce significant diseases in both captive and wild reptiles; therefore, there is a need to develop rapid diagnostic tests that will aid disease surveillance and reporting in order to maintain safe biosecurity measures and reduce spread.

EPIDEMIOLOGY

Epidemiological studies of HVs in wild reptiles could be challenging due to a lack of sensitive diagnostics for the detection of unknown or novel species, especially in resource-limited regions. HV infections are commonly characterised by non-specific clinical signs in most reptiles, thus making diagnoses on the basis of clinical signs alone difficult. An exception to this is FP, in which the presence of cutaneous tumours gives an indication of the disease; hence, more FP-associated HV data have been reported in recent decades (**Figure 2**; **Supplementary File 2**). Even so, the complete disease impact on wild populations could be currently underestimated due to the underreporting of outbreaks, sampling bias and poor monitoring systems. For instance, there is a paucity of information for FP epidemiology

TABLE 2 | Clinical presentations, gross, and histological lesions of reptilian HV species.

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
ChHV-1	• Green sea turtles (*Chelonia mydas*)	• Benign papular lesions on the neck and flippers • Spreading grayish patches to large areas of the epidermal surface • Death may occur	• Benign Papules • Spreading gray patches	• Intranuclear inclusions found in epidermal keratinocytes	(54, 95, 96)
ChHV-2	• Pacific pond turtles (*Clemmys marmorata*)	• Lethargy • Anorexia • Muscular weakness • Coma • Subcutaneous haemorrhages • Death	• Hepatomegaly • Pallor of kidney • Subcutaneous Petechial and ecchymotic haemorrhages	• Hepatic necrosis • Intranuclear inclusion bodies • Lymphocytic aggregation in liver, kidney, and spleen • Moderately hyperplastic spleen	(55)
ChHV-3	• Painted turtles (*Chrysemys picta*)	• Abscessation • Death	• Pulmonary edema • Friable and greenish-brown liver • Distended gall bladder • Congested spleen • Shell rot lesions on plastron	• Foci of necrosis on the liver and infundibular septa • Hepatocytes containing Eosinophilic intranuclear inclusions • Granulocytic and mononuclear infiltrations	(56)
ChHV-4	• Argentine tortoise (*Geochelone chilensis*)	• Acute death • Nasal discharge • Ocular discharge • Regurgitation • Anorexia • Lethargy • Necrotizing stomatitis	• Necrotizing lesions • Serous atrophy of fat • Pale liver	• Diffuse area of necrosis in mucosal epithelium • Accumulation of necrotic cellular debris and fibrin • Infiltration of Inflammatory cells • Eosinophilic intranuclear inclusions within degenerating epithelial cells and other tissues • Vacuolar degeneration of hepatocytes	(57)
ChHV-5	• Green sea turtle (*Chelonia mydas*) • Loggerhead sea turtle (*Caretta caretta*) • Hawksbill turtle (*Eretmochelys imbricata*) • Leatherback turtle (*Dermochelys coriacea*) • Olive ridley sea turtle (*Lepidochelys olivacea*) • Kemp's ridley sea turtle (*Lepidochelys kempii*) • Flatback turtle (*Natator depressus*)	• Tumours on the inguine, tail, flippers, axillae, chin, neck, eyelids, corneas, carapace and plastron	• Single to multiple raised cutaneous masses that are verrucous, smooth, sessile or pedunculated • Ulcerated and necrotic large masses • Pigmented cutaneous tumours • Spherical, smooth, firm, white, or gelatinous and translucent nodules in the lungs, kidneys, liver, heart, and gastrointestinal tract	• Papillary epidermal and dermal hyperplasia • Orthokeratotic hyperkeratosis • Hypertrophied epithelial cells overlying vascularized fibrous stroma • Epithelial necrosis and multifocal areas of ballooning degeneration • Lymphocytes and plasma cells infiltrations • Melanophores within the masses • Eosinophilic intranuclear inclusions	(97)
ChHV-6	• Green sea turtles (*Chelonia mydas*)	• Gasping • Buoyancy abnormalities • Inability to dive • Lethargy • Caseous exudate covering the eyes, glottis and trachea • Death	• Emphysematous areas in the lungs • Caseous exudate in the eyes, glottis and trachea • Multifocal raised white nodules in the liver	• Necrotic lesions in the glottis, tracheal and lungs • Periglottal accumulations of necrotic cellular debris and fibrin • Infiltrations of heterophils, lymphocytes, and plasma cells in periglottal submucosa • Periglottal and tracheal epithelial proliferative and/or squamous metaplastic changes • Syncytial giant cells in tracheal mucosa and major airways of lungs • Thickened interstitium	(98)

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
				• Hypertrophic and hyperplastic cells with enlarged vacuolated nuclei lining the airways • Amphophilic intranuclear inclusions	
TeHV-1	• Horsfield tortoises (*Testudo horsfieldii*) • Pancake tortoises (*Malacochersus tornieri*)	• Cervical extension • Laboured breathing • Respiratory murmur • Oral and nasal discharge • Reddish-white fibrinous coating of the tongue • Death	• Yellowish-white pseudomembrane in the mouth, pharynx and glottis • Hepatomegaly and ecchymotic liver • Pseudomembrane formation in the stomach	• Diffuse areas of degeneration and necrosis in tongue and pharynx and larynx • Necrotic cellular debris and fibrin accumulation • Inflammatory cells infiltrations • Eosinophilic or amphophilic inclusion bodies	(99–101)
TeHV-2	• Desert tortoises (*Gopherus agassizii*)	• Anorexia • Lethargy • Necrotizing stomatitis	• Oral plaques	• Eosinophilic and amphophilic intranuclear inclusions in superficial epithelial cells • Thick coagulum over the epithelial surfaces of the mouth, pharynx, and trachea • Infiltration of heterophils, lymphocytes, plasma cells, and macrophages • Granulation of oropharyngeal tissue following epithelial loss	(44)
TeHV-3	• Greek Tortoises (*Testudo graeca*) • Hermann's Tortoises (*Testudo hermanni*)	• Nasal and oral discharges • Rhinitis • Dyspnoea • Conjunctivitis associated with blepharospasm • Diphtheroid-necrotizing stomatitis • Glossitis • Pharyngitis • CNS involvement (Circling, head tilt, lethargy, circling, paralysis and incoordination) • Deaths	• Stomatitis with yellowish oral plaques • Rhinitis with foamy nasal discharge • Conjunctivitis	• Oesophageal hyperplasia • Hyperplasia and hyperkeratosis in the oral mucosa • Sloughing of the epithelial cells and multifocal erosion • Glottal epithelial ulceration, hyperplasia and necrosis • Heterophilic pustules. • Amphophilic intranuclear inclusion bodies • Heterophilic bronchitis and pneumonia • Nuclear degeneration changes of the hepatocytes • Ballooning degeneration renal and digestive organs	(14, 78, 102, 103)
TeHV-4*	• Bowsprit tortoise (*Chersina angulata*) • Leopard tortoise (*Stigmochelys pardalis*)	• Asymptomatic in some cases. • Respiratory distress • Increased salivation	• No data	• No data	(43, 104)
LGRV	• Loggerhead sea turtles (*Caretta caretta*)	• Moribund state • Lethargy and quadriparesis • Emaciation • Abnormal gait • Death	• Colon impaction • Fibrinonecrotic colitis • Linear ulcers around the base of the phallus • Multifocal ulcers along the mucocutaneous junction of the eyelids • Circumferential ulcer around the entire mucocutaneous junction of the cloaca • Ulcerative gastritis	• Epithelial hyperplasia. • Ballooning degeneration and syncytial cell formation within basal layers of the epithelium • Intranuclear eosinophilic inclusion bodies • Heterophilic inflammation	

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
LOCV	• Loggerhead sea turtles (*Caretta caretta*)	• Moribund state • Lethargy, bradycardia, hypoventilation, and aspiration pneumonia. • Death	• Deep multifocal ulcers around the rostral aspect of the tongue • Multifocal pale cutaneous plaques with erythematous borders on the ventral neck region • Tenacious exudates covering some plaques • Liver pallor	• Eosinophilic intranuclear inclusion bodies • Necrosis of the epithelium and extend into the underlying lingual collagen • Heterophilic inflammation • Epidermal hyperplasia • Hyperkeratosis • Intraepithelial pustules • Multifocal serocellular crust • Sloughed epithelial cells in the airways	(42)
EBHV-1*	• Blanding's turtles (*Emydoidea blandingii*)	Asymptomatic	• No data	• No data	(39)
GlyHV-1*	• Bog turtles (*Glyptemys muhlenbergii*)	Asymptomatic	• No data	• No data	(37)
GlyHV-2*	• Wood turtles (*Glyptemys insculpta*)	Asymptomatic	• No data	• No data	(37)
EmyHV-1	• Eastern river cooter (*Pseudemys concinna*) • Northern map turtle (*Graptemys geographica*) • Painted turtles (*Chrysemys picta*)	• Weakness • Frothy nasal discharge • Acute death	• Dark red, wet, and heavy lungs • Thickened, wet, and gelatinous cranial aspect of the lungs • Trace amount of watery fluid in the trachea • Diffusely tan, and slightly rounded lobular edges of the liver	• Hepatic lipidosis • Intranuclear inclusion bodies • Necrotic lesions in the lungs, liver and spleen • Granulocytic and lymphocytic interstitial infiltrations • Acute congestion with multifocal haemorrhage	(28, 105)
EmyHV-2*	• Bog turtle (*Glyptemys muhlenbergii*) • Spotted turtles (*Clemmys guttata*)	Asymptomatic	• No data	• No data	(37)
TerHV-1*	• Eastern box turtles (*Terrapene carolina carolina*)	• Lethargy • Dehydration • Dyspnoea • Moribund state with fibronecrotic stomatitis and cloacitis • Conjunctivitis • Blepharoedema • Death	• No data	• Necrosis, ulceration and syncytia formation of the pharyngeal mucosal epithelium • Eosinophilic to amphophilic intranuclear inclusions	(40)
TerHV-2	• Eastern box turtles (*Terrapene carolina carolina*)	• Papillomatous skin lesions • Anorexia	• Cutaneous papillomas	• Papillary hyperplasia of the epithelium • Infiltrations of lymphocytes, plasma cells, and heterophils • Epithelium covered by keratin and cell debris	(38)
Pelomedusid HV-1*	• West African mud turtles (*Pelusios castaneus*)	• Asymptomatic	• No data	• No data	(41)
IgHV-1	• Green iguana (*Iguana iguana*)	• Acute death	• Thin body • Generalized muscle wasting • Loss of fat store	• Hepatocellular necrosis • Hepatic syncytia • Eosinophilic intranuclear inclusions • Stomach and intestinal ulceration and necrosis • Acute renal tubular necrosis • Splenic lymphoid atrophy or hypoplasia	(33, 50, 51)

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
IgHV-2	• San Esteban Chuckwalla (*Sauromalus varius*)	• Acute death	• Haemorrhage in the lung • Congestion of airway • Pale liver	• Diffuse hepatic necrosis, eosinophilic intranuclear inclusions • Multifocal necrosis of the spleen • Interstitial infiltrations of muscles by mononuclear leucocyte • Fibrosis of muscle and gingiva	(34)
Gerrhosaurid HV-1	• Sudan plated lizard (*Gerrhosaurus major*)	• Glossal stomatitis • Severe dyspnoea	• Raised and tanned periglottal tongue • Little body fat	• Glottal trachea of granulocytic and lymphocytic inflammation with erosion of overlying epithelium	(47)
Gerrhosaurid HV-2*	• Black-lined plated lizard (*Gerrhosaurus nigrolineatus*)	• Labial stomatitis	• No data	• No data	(47)
Gerrhosaurid HV-3*	• Sudan plated lizard (*Gerrhosaurus major*)	• Chronic labial proliferative and ulcerative growth	• No data	• No data	(47)
VHV-1	• Green tree monitor lizards (*Varanus prasinus*)	• Proliferative and Ulcerative stomatitis/gingivitis • Squamous cell carcinomas	• Small white chalky plaques in the coelomic membrane, thoracic musculature, liver, kidneys, heart, and joints fascial plane • Gingival proliferation • Mucosal hyperplasia • Fibrinous exudate on the serosa of the gall bladder • Oral villous-like proliferation with patches of focal erythema	• Mucosal epithelial proliferation • Severe pulmonary, myocardial, hepatic, and renal vascular thrombosis • Sloughed tubular endothelial cells • Gingival necrosis • Hepatic lipidosis • Hepatic and renal amyloidosis	(48)
VHV-3	• Monitor Lizards (*Varanus spp.*)	• Acute death	• Yellow-tan or white viscous material and white, thick material in the intestine and distal colon, respectively • Multiple soft, white particles (2–3 mm) in intestinal tract • Diffuse pale-brown liver with multiple flat, tan pinpoint foci on the capsular surface	• Acute, multifocal, coagulative necrosis in the lamina propria of the small intestine • Acute, multifocal hepatocellular coagulative necrosis • Eosinophilic intranuclear inclusions in the small intestine and liver	(49)
HeHV-1	• Gila monster (*Heloderma suspectum*)	• Intraoral mass • Loss of weight	• Gingival nodule • Muscle atrophy	• Anastomosing epithelial cords • Proliferative gingival tissues • Eosinophilic and birefringent material within mass	(25)
Elapid HV-1	• Siamese cobra (*Naja naja kaouthia*)	• Thick tenacious venom (low grade venom)	• Enlarged venom gland • Thick venom exudates	• Venom glands are lined by degenerated epithelial cells • Mononuclear cell infiltration of gland subepithelium • Debris, degenerated cells and venom in the lumina of glands • Intranuclear inclusions	(106)
Opheodrys HV-1	• Smooth green snakes (*Opheodrys vernalis*)	• Oropharyngeal squamous cell carcinoma	• Pale tan, multinodular masses on oropharyngeal mucosa • Brown friable accumulations on tumour surface	• Distorted oropharyngeal mucosa and submucosa by epithelial neoplasm • Islands of neoplastic epithelial cells containing keratin cores • Anisocytosis and anisokaryosis of neoplastic epithelial cells	(52)

(Continued)

TABLE 2 | Continued

Species	Reported host	Clinical presentation	Gross lesion	Histopathology	References
				• Squamous differentiation, keratin pearls, prominent intercellular bridges • Heterophilic inflammation and surface compact keratin layers	
CrHV-1	• Saltwater crocodiles (*Crocodylus porosus*)	• Conjunctivitis-pharyngitis (CP).	• Reddening and swelling of the conjunctivae of the eyelids and nictitating membrane • Cornea opacity and rupture • Fibrinocaseous conjunctival, lingual and oropharyngeal exudates	• Epithelial Hyperplasia, erosion, or ulceration of the conjunctiva, pharynx and larynx with cellular infiltrations • Lymphocyte, heterophil, and macrophage infiltrations of cornea, iris, and conjunctival, pharyngeal and laryngeal epithelium	(53, 107)
CrHV-2	• Saltwater crocodiles (*Crocodylus porosus*)	• Conjunctivitis-pharyngitis (CP) • Concurrent skin ulcers. • Systemic lymphoid proliferation and encephalitis (SLPE) • Lymphnodular skin (LNS)	**CP** • Gross lesions of CP syndrome as described above **SLPE** • Poor body condition • Splenomegaly. • Pulmonary edema **LNS** • Pale, soft, raised, well-delineated foci on lateral abdominal scales with occasional ulcerated surface covered in caseous exudate • Pale pink soft glistening tissue between the epidermis and deep dermal collagen • Enlarged tonsils with multinodular appearance • Discrete soft white foci in the subepithelial tissue of the conjunctiva • Multinodular swelling of the cloacal mucosa • Discrete white soft foci in the parenchyma of the myocardium, liver, or kidney	**CP** • Histological lesions of CP syndrome as described above **SLPE** • Lymphohistiocytic and macrophage infiltration of pulmonary septae, hepatic periportal regions, pancreatic interstitium, gastrointestinal submucosa, pericardium, epicardium, iris, wall of large blood vessels and brain • Hyperplastic lymphocytic conjunctivitis **LNS** • Expansion and displacement of collagen of the superficial and mid-dermis by intense multinodular mononuclear cell infiltrate • Epithelial hyperplasia of the tonsils will lymphocytes and macrophage infiltrations • Dense lymphohistiocytic aggregates of myocardium, liver, or kidney	(53, 107)
CrHV-3	Freshwater crocodiles (*Crocodylus johnstoni*)	• Systemic lymphoid proliferation	• Gross lesions of SLPE described above	• Histological lesions of SLPE described above	(53, 107)

ChHV, Chelonid herpesvirus; TeHV, Testudinid herpesvirus; LGRV, loggerhead genital-respiratory herpesvirus; LOCV, loggerhead orocutaneous herpesvirus; EBHV, Emydoidae herpesvirus; GlyHV, Glyptemys herpesvirus; EmyHV, Emydid herpesvirus; TerHV, Terrapene herpesvirus; IgHV, Iguanid herpesvirus; VHV, Varanid herpesvirus; HeHV, Helodermatid herpesvirus; CrHV, Crocodylina herpesvirus.
**To the best of our knowledge, the gross or histological lesions of some novel viruses have either not been detected, or were reported while this manuscript was being written.*

at the pelagic phase of life in sea turtles as most studies are biassed towards sampling nearshore juveniles and adult females at foraging grounds or nesting beaches. Nevertheless, wildlife workers and researchers who, despite numerous challenges, have provided considerable epidemiological data targeted at conservation efforts towards endangered species should be commended. An overview of some of the epidemiological information including the prevalence and demography of both wild and captive reptilian HVs is discussed in this section.

Herpesviruses are linked to different diseases of marine turtles, including FP, LETD and GPD (54, 82). FP is a debilitating disease characterised by the development of tumours (119, 120). Depending on the location of the tumours, FP can have detrimental effects (109, 121). On the basis of prevalence and distribution, Tagliolatto et al. reported a prevalence rate of 43% for FP in green turtles captured in a foraging area in south-eastern Brazil (121). Adnyana et al. recorded 22% overall prevalence in green turtles in Indonesia and also observed that the prevalence rate of FP was higher among turtles from waters adjacent to densely populated regions compared to those collected from waters remote from urbanised regions of Indonesia (122). These findings indicate that the epidemiology of FP in marine turtles vary between geographical regions and may be linked to anthropogenic activity. This theory is supported by the findings in another study, which attributed the variation of FP prevalence to environmental cofactors that vary among local habitats (123). A study associated the geographical distribution of FP with the genomic variation of HVs in marine turtles, and observed four forms of the virus corresponding to Atlantic Ocean, west Pacific, mid-Pacific, and east Pacific (124). A similar study conducted in Australian waters identified different genotypes along the east coast of Queensland. Such differences in strains may also effectuate different levels of pathogenicity between strains (76, 77) and account for variation in reported

FIGURE 6 | Fibropapillomatosis in green turtle (*Chelonia mydas*). Photo by Dr. Karina Jones.

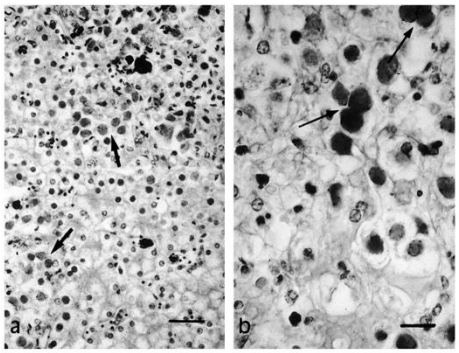

FIGURE 8 | Necrotic foci **(a)** and syncytial formation **(b)** in HV infected hepatocytes of a tortoise (*Testudo horsfieldii*). "Adapted from Hepatitis Associated with Herpes Viral Infection in the Tortoise (*Testudo horsfieldii*)" by Hervás et al. (112). Copyright 2021 by John Wiley and Sons. Reprinted with permission.

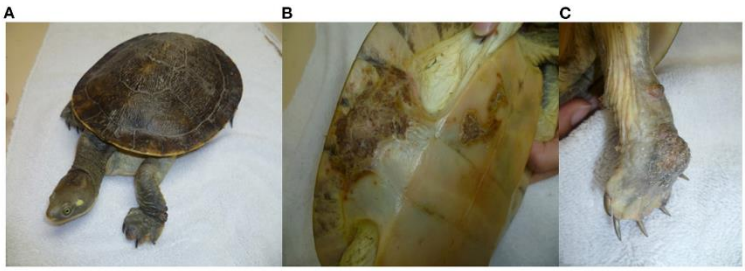

FIGURE 7 | Herpesvirus infection in freshwater turtle (*Emydura macquarii krefftii*) presented with proliferative and ulcerative lesions of the skin **(A)**, proliferative and crusted lesions on the bridge of the shell **(B)**, and proliferative lesion on the palmar aspect of the right forefoot **(C)**. "Adapted from Herpesvirus in a captive Australian Krefft's river turtle (*Emydura macquarii krefftii*)" by Cowan et al. (110). Copyright 2021 by John Wiley and Sons. Reprinted with permission.

prevalence in different regions. Also, given that the immune system of reptiles is dependent on temperature (125, 126), the variation in the prevalence rates of reptilian HVs across regions could be associated with differences in regional climate types. Comprehensive reviews of the epidemiology of FP in marine turtles have been documented elsewhere (73, 97, 127–129). The host immune status influences the clinical course of a disease, as immuno-deficient populations are more likely to succumb to disease outbreaks. Serosurveys have been conducted to determine the immune status of populations and to provide evidence of past and ongoing HV infections (130, 131). Seroepidemiological studies in three localities in Florida revealed high anti-ChHV seroprevalences (up to 100%) in both FP and non-FP sea turtles (81, 132). Contrastingly, seropositivity to ChHV-5 was dependent on the tumour status in turtles from Hawaii (133). This variation was mainly attributed to differences in the pathogenicity of ChHV-5 subtypes from the two regions (133). In another report, an epizootic of LETD in confined juvenile green sea turtles resulted in 8 to 38% mortality, thus posing significant conservation and management concerns (98, 134). The LETD impact on free ranging sea turtles has not been investigated; however, seroprevalence rates of 13% and 22% were reported in two studies, respectively (134, 135).

Similarly, HV infections are causing increasingly significant concerns in non-marine chelonians (27, 39). Herpesviruses have been implicated as the cause of severe clinical signs and acute death in terrestrial and freshwater turtles (**Table 2**) (28, 40, 105, 110, 111, 136). Although, HVs have been associated with latent infections in their natural hosts, infections in young, immunosuppressed or non-adapted hosts could result in the development of significant diseases (137). Therefore, monitoring the disease impact on both wild and captive endangered species has become pertinent. In an epidemiological study conducted in Tennessee and Illinois, USA, 128 of 409 free-ranging eastern box turtles (*Terrapene Carolina Carolina*) tested positive for TeHV-1 using TaqMan quantitative PCR, and the detection rate varied widely between seasons (138). Another study reported 48.3% prevalence of HV infections in endangered populations of bog (*Glyptemys muhlenbergii*), wood (*G. insculpta*), and spotted (*Clemmys guttata*) turtles in the northeastern United States (37). Furthermore, tortoise HVs have been associated with high mortality and morbidity (104, 113, 139–141). Different HV species were identified to cause the death of a large number of pancake (*Malacochersus tornieri*), Horsfield (*Testudo horsfieldii*), Hermann's (*Testudo hermanni*), and Egyptian tortoises (*Testudo kleinmanni*) during spontaneous outbreaks in Japan, Italy and Germany, respectively (99, 102, 142). Species dependent susceptibility to HV was reported in a tortoise colony in which *T. graeca* and *T. horsfieldii* appeared to be unaffected by the HV species that caused the death of other tortoises in the same colony (113). A possible explanation could be that the causative HV species is well-adapted in these tortoises and they could be transmitting the virus to naïve or non-adapted tortoises. Of the four tortoise HV species (TeHV1-4), TeHV-3 appears to be the most pathogenic and frequently described, causing lethal disease in different tortoise species (14, 142–145). In a recent assessment of the incidence of chelonian HVs in Europe, more than half (54%) of all the detected chelonian viruses were TeHV-3 (146). Again, seroprevalence rates of 27% and 31% were reported for TeHV-1 and TeHV-3, respectively, in different populations of desert tortoises in California (147, 148). Despite the significance of HV infections, we observed that the disease is still grossly under-studied in some countries (**Table 1**). Thus, insufficient data and underreporting have made it difficult to assess the geographical patterns of the HV epidemiology in non-marine chelonians and other reptiles.

Herpesviruses have also been described in various species of squamates and crocodilians (25, 47, 48, 52, 106, 116–118, 149). A recent outbreak of a lethal HV infection in a private facility housing 127 snakes resulted in the death of all 71 horned vipers at the premises after a brief illness (150). An earlier study also implicated HV in the death of some boa constrictors within the first year of life (151). Herpesvirus-induced deaths have been reported in different species of lizards with case fatalities nearing 100% (33, 49, 152). As stated earlier, HV infections in crocodiles are associated with CP, SLPE and LNS syndromes (53). Another study strongly linked HV infection to SLPE and CP syndromes in farmed Australian saltwater crocodiles, with the highest prevalence rates of 94 and 54%, respectively (107). Crocodiles are intensively farmed for commercial purposes in Australia; therefore, the occurrence of HVs in crocodiles has both epidemiological and economic implications (53, 153).

Finally, we extracted a total of 130 articles, of which 39% (51 articles) and 32% (41 articles) were studies that investigated HVs in marine turtles and tortoises, respectively. A total of 21 (16%) studies investigated HVs in freshwater turtles. HVs were least studied in lizards (8%; 11 articles), snakes (3%; 4 articles) and crocodiles (2%; 3 articles) (**Figure 2**). The scant studies of HVs in some reptilian species since the 1970s could be attributed to the unavailability of reagents or sensitive diagnostic assays required to investigate reptilian diseases in remote areas or the lack of interest to investigate HVs in reptiles because of their relatively low socio-economic importance. Therefore, future efforts should be directed towards enhancing collaborations among government agencies, researchers and wildlife workers with a view to creating awareness, increasing access to reagents and sensitive assays, and ultimately conserving endangered reptiles.

DIAGNOSIS

A timeline of reptilian HV diagnosis showed that traditional assays including histopathology, virus isolation (VI) and electron microscopy (EM) have been the mainstays in the diagnosis of reptilian HVs (**Supplementary File 4**). Many studies have reported the use of these techniques since the 1970s for the investigation of reptilian HVs. A breakdown of the number of studies that have used these methods to detect reptilian HVs is shown in **Figure 3**. Molecular diagnosis of reptilian HVs started two decades ago and has been used increasingly since then (**Figure 3**; **Supplementary File 4**). The advent of molecular diagnostic techniques has provided insight into the genetic characteristics and the phylogenetic relationship of most reptilian

HVs. This section highlights some important characteristics of the various techniques used in the diagnosis of reptilian HVs.

Diagnosis of reptilian HVs is tentatively made on the basis of patient history, clinical signs, and gross and histological lesions (16). However, this is not always the case, as host-adapted HVs can cause subclinical, mild or latent infections in their natural hosts, and the demonstration of intranuclear inclusions is not pathognomonic of reptilian HV infections (154). Intranuclear inclusions are frequently associated with other reptilian viruses including adenoviruses and papillomaviruses (155–157). Earlier researchers used EM to confirm the presence of reptilian HV infections by demonstrating the ultrastructure of the viral particles in fixed, cut and stained sections of tissue samples (51, 54, 98, 106, 116, 136, 140, 158). More recently, EM has been used to confirm a necrotic hepatitis associated with HV infection in a tortoise with no clinical signs or lesions in the respiratory tract, oral cavity or other organs (112). The need for high technical capacities and the high cost of electron microscopes limit the use of EM for epidemiological and diagnostic purposes especially in resource-limited areas. Despite these limitations, EM remains a powerful detection tool in most high-class virology laboratories.

Reptilian HVs have been isolated in cell culture and identified on the basis of their cytopathic effects (50, 98, 107, 159). For instance, tortoise HVs were isolated from pharyngeal swabs, trachea, kidney, oesophagus, tongue, stomach, and intestine, and caused cytolysis and rounding of cells in terrapene heart cells (TH-1) (160). In another study, detachment and foci of enlarged, rounded, refractile cells were produced following inoculation of tissue and swab supernatants in turtle heart cells (142). ChHV-5, which historically has been resistant to replication in conventional cultures, produced *de novo* ballooning degeneration and eosinophilic intranuclear inclusion in plugs and organotypic skin cultures (89). This observation implies that ChHV-5 remains latent in conventional cultures and requires replication of the turtle skin to grow *in vitro* (89). Aside from the fact that CPE are not obtained for non-cytopathic viruses, cell culture is susceptible to both chemical and biological contaminations, which in turn affect its sensitivity and specificity. Also, diagnostic turnaround could be delayed for slow-growing viruses. Therefore, it should not be solely relied upon for the epidemiological investigations of HVs.

Following primary infections in reptiles, a strong non-specific (innate) immune response that includes lysozymes, leukocytes, natural antibodies (NAbs), antimicrobial peptides, and the complement pathway, is quickly stimulated (126, 161). No specific information is currently documented about adaptive cell mediated immunity to HV infections. Unlike mammals, in reptiles a less robust and slower humoral response (IgA, IgD, IgM, and IgY) is stimulated after the innate immune system is activated (126, 154). In tortoises, neutralising antibodies to HV infection were detectable in serum at least 4 weeks post-exposure (162). These serum neutralising antibodies did not appear to confer immunity to reinfection or recrudescence (78). Later seroconversion was observed (four months to one year) in green turtles (*Chelonia mydas*) that were experimentally infected with ChHV (81, 132). Generally, the detection of anti-herpesvirus antibodies in a single sample could indicate previous or latent infection, while rising antibody titre in paired samples collected at least 6 weeks apart indicates active infection (154, 163). Humoral antibodies are detected by serological assays such as serum neutralisation (SN) tests, ELISA, and immunoperoxidase (IP) assays (132, 164–166). The SN test is considered the reference test for anti-herpesvirus antibody detection but has limitations such as a delayed turnaround, inherent assay arduity and the requirement for standard isolates (162). ELISAs with high sensitivity and specificity have been developed and deployed in various seroepidemiological studies (44, 81, 132, 147, 162, 166). However, a high degree of cross-reactivity that potentially affects assay specificity has been demonstrated among different tortoise HV isolates used as antigens in the ELISA (147, 162). Cross-reactivity could also occur in other reptilian HVs that share similar antigenic epitopes, giving false positive results and, thus, leading to unnecessary post-exposure interventions. Overall, serological diagnostic techniques are not useful for the early diagnosis of reptilian HVs because of the delay in antibody response and the need for paired serum sample collection weeks apart with accurate timing. However, it can play an important role in retrospective studies and in the diagnosis of latent or asymptomatic patients.

Recent epidemiological studies have largely relied on molecular methods to identify potential genetic and environmental risk factors associated with reptilian HVs (24, 80, 138, 167–169). Species-specific PCR-based assays targeting specific gene segments of reptilian HVs have been developed and validated (78, 170). Lindeman et al. developed two quantitative PCR assays and recorded a detection limit as low as 1 viral copy per reaction using primers that targeted the EBHV-1 specific segment of DNA polymerase gene (U_L30) (39). In another study, two TaqMan PCR assays developed to target the U_L30 gene of TerHV-1 detected 10 viral copies per reaction (171). Conventional and heminested PCR assays using tortoise HV-specific primers have been developed with assay sensitivity of 10^3 and 10^1 DNA copies, respectively (172). Alternatively, consensus PCR techniques developed by VanDevanter et al. have been employed for the molecular screening and novel detection of reptilian HV species (39, 43, 100, 173–177). Although the molecular assays for the diagnosis of reptilian HVs have demonstrated excellent performance, their use still presents a major challenge in remote areas due to high cost, complexity of instrumentation, aseptic technique requirement and the need for electricity to operate PCR machines.

In order to accurately estimate the magnitude and scope of a disease outbreak or occurrence, case definition (that is, standard criteria for categorising diseases) would need to be established. One of the ways to achieve this is to make available rapid, sensitive and affordable assays for confirming the presence of diseases. Rapid diagnostic immunoassays that use lateral flow or chromatographic strategies should be developed for the rapid diagnosis of reptilian HV infections in the field or point of care (POC) settings. This approach could overcome some of the above-mentioned diagnostic challenges, especially in low resource areas. However, the use of lateral flow immunoassays for viral detection in other species have been marred by low and varying sensitivities (178–181). Sensitive molecular-based

rapid assays are relatively expensive and yet to be employed for the diagnosis of reptilian HVs (182–185). We would propose an ultrasensitive format that combines PCR and immunoassay but then it can be argued that such a laboratory-based system is less rapid and has limited use in low-class laboratories (186, 187). Rapid detection techniques such as Microfluidic chip immunoassay and Smartphone-based rapid telemonitoring system (SBRTS) are fast becoming powerful tools in the diagnosis of viral infections (188–196). Of particular interest, is the SBRTS that combines biosensor and smartphone functionalities to produce a rapid, sensitive and cheap detection system (197). SBRTS has an average turnaround of 30 min, overcomes inherent problems associated with sample handling and preparation, and can remotely monitor and report data on disease occurrence, thus making it suitable for use in resource-limited countries (193, 197). This assay if employed could tick all the boxes for the epidemiological investigation and reporting of reptilian HVs.

Herpesvirus diagnostic and epidemiological data should be interpreted with prudence because of the possible influence of coinfection variables that could cause the reactivation of seemingly latent HV infections. For instance, some studies have reported the detection of co-pathogens in reptiles showing clinical signs, some of which are typical of HV infections (27, 38, 146, 168, 173, 198, 199). These observations imply that the detection of HVs may not be the actual cause of the current disease, but because the immune system is compromised by other pathogens, the HVs recrudesce and become easier to detect. Both latency (decreases apparent prevalence and significance) and coinfections (increase apparent prevalence and may also falsely assign the clinical signs to the HV) will have an influence on the disease picture. Therefore, we recommend that biosecurity and conservation measures should include a multiplex pathogen detection model whenever possible in order to fully assess the health of reptilian populations.

TREATMENT, PREVENTION, AND CONTROL

Surgical excision, carbon dioxide (CO_2) laser surgery and cryosurgery are some of the commonly used therapeutic strategies for the management of HV-associated tumours (25, 110, 111, 120, 200–202). High rates of recurrence and the risk of secondary bacterial infections have greatly reduced the efficacy of surgical excision (200, 203). CO_2 laser surgery, which combines laser excision and ablation of tumours, has shown improved intraoperative and postoperative outcomes and is therefore the treatment of choice (120, 201, 204). Non-surgical approaches including electrochemotherapy (ECT) and photodynamic therapy (PDT) with no known recurrence have recently been employed as alternatives in the treatment of FP (205, 206).

Several authors have recommended the use of acyclovir complemented by fluid and antibiotic therapies for the effective treatment of tortoise HV infection (143, 207–209). Marschang et al. showed that acyclovir and ganciclovir effectively inhibited HV replication *in vitro* at a single dose or repeated daily dose of 25 or 50 μg/mL (142). Similarly, the *in vitro* activities of acyclovir and ganciclovir were recently tested and shown to be effective against TeHV-3; however, the safety of these drugs is yet to be demonstrated in tortoises (210). Based on the toxicity (on liver and kidney cells) and other biochemical data, this same study showed that eprociclovir is not suitable for use as anti-TeHV-3 in Hermann's tortoises and further *in vivo* assessment of other potential antiviral drugs was recommended (210).

Recently, an autogenous vaccine therapy was proposed and used for the treatment of HV-associated papillomatosis in Williams' mud turtle (*Pelusios williamsi*) (111). The autogenous vaccine, which was aseptically prepared from excised fresh tissue induced substantial areas of necrosis of the papillomatous lesions, thus indicating the efficacy of the vaccine (111). Autogenous vaccines potentially contain relevant neoantigens that comparatively improve their efficacy (211). However, their use could be limited by lack of sufficient tumours (in patients) needed to produce adequate vaccine doses. Also, no standard protocol exists for autogenous vaccine production and delivery, and patients' tumours may progress beyond the intervention stage before the vaccine becomes ready for delivery. Allogeneic vaccines on the other hand, can overcome some of the aforementioned challenges; however, they may lack the advantageous self-neoantigens (211). In the past, an inactivated vaccine was evaluated against tortoise HV without success as no significant rise in antibody was detected in vaccinated tortoises after 369 days post vaccination (160). DNA or mRNA based vaccines have the capacity to induce both humoral and cellular immune responses and have shown promising outcomes against some animal and human diseases (212–215). Although vaccine research and development could be costly, laborious and time-consuming, the nucleic acid vaccines hold the potential to significantly reduce HV-associated losses in captive collections and wild reptiles of conservation concerns.

Prevention is of utmost importance in the management of reptilian HV infections, since death may still occur following therapeutic interventions and recovered animals remain latent carriers (143, 163). Unfortunately, there are no established preventive or control measures for HV infections in wild populations of reptiles (200), which consequently presents a major conservation challenge. Environmental factors including degraded water quality caused by pollutants, increased water temperature, natural biotoxins, and high dietary arginine concentrations due to microalgae bloom have arguably been linked as cofactors in the development of FP in sea turtles (73, 119, 216–221). Therefore, adopting conservation actions needed to regulate water and species management, as well as regulating human activities leading to climate change, would be sensible.

In captive reptiles, quarantine procedures and adequate testing of new acquisitions are strongly recommended (153, 163, 167). All previously infected or HV seropositive animals should be treated as latent carriers and potential shedders to naïve populations, as factors including stress, bad husbandry, illness or immunosuppression could reactivate the virus (14, 163, 167, 200). Generally, strict hygiene practises and adequate biosecurity should be followed in all facilities housing reptiles (162, 172, 222–224).

AUTHOR CONTRIBUTIONS

GO and EA conceived and designed this review. GO wrote the manuscript, analysed the data, and prepared figures and tables. EA, PH, and DW contributed to the concept and reviewed drafts of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.642894/full#supplementary-material

Supplementary File 1 | Literature review search terms and strategies.

Supplementary File 2 | Data extraction for literature review.

Supplementary File 3 | Co-occurrence map of all keywords from 1975 to 2021. We found a total of 93 keywords with a minimum of 5 occurrences. Node size represents the number of occurrences of each keyword. The lines denote the total link strength between keywords, and it is proportional with the width. The line colours denote five different keyword clusters. The clusters gave an indication of the main areas research interest of reptilian HVs.

Supplementary File 4 | A timeline of reptilian HV diagnosis. A review of articles conducted from 1972 to 2020 showed that VI, EM, histopathology, and serologic assays have been used for the detection of reptilian HVs since 1972. Nucleic acid detection assay (PCR) was first reported in 1998 for detection of ChHV-5 in fibropapillomas. Events were chosen based on information from the extracted articles (**Supplementary File 2**).

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ORIGINAL ARTICLE

Development of subfamily-based consensus PCR assays for the detection of human and animal herpesviruses

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Abstract
Consensus PCR assays that can be used to sensitively detect several herpesvirus (HV) species across the different subfamilies were developed in this study. Primers containing degenerate bases were designed to amplify regions of the DNA polymerase (DPOL) gene of alpha- and gamma-HVs, and the glycoprotein B (gB) gene of beta-HVs in a singleplex, non-nested touchdown PCR format. The singleplex touchdown consensus PCR (STC-PCR) was used to amplify the DNA of eight human and 24 animal HVs. The assay was able to detect the lowest DNA dilution of 10^{-5} for alpha-HVs and 10^{-3} for beta- and gamma-HVs. In comparison, lowest detection limits of 10^{-5}, 10^{-3}, and 10^{-2} were obtained for alpha-, beta-, and gamma-HVs respectively when a nested PCR was used. The findings in this study suggest that the STC-PCR assays can be employed for the molecular surveys and clinical detection of novel and known HVs.

Keywords Herpesvirus · Consensus PCR · DNA polymerase gene · Glycoprotein B gene

Introduction

Herpesviruses (HVs) are known to have a wide host range, infecting both vertebrate and invertebrate species [1, 2]. The virus is made up of a linear, monopartite, double-stranded DNA genome that encodes up to 300 genes and ranges from 124 to 241 kbp in length [3]. Herpesviruses are divided into three subfamilies, the *Alpha*-, *Beta*-, and *Gamma-herpesvirinae* on the basis of biological and molecular properties [2]. A common feature among all of the sub-groups of HVs is their ability to cause latent infection in infected hosts, which can be reactivated to cause serious illness in immunocompromised hosts [2]. Clinical diseases associated with active or recrudescent HV infections vary according to the hosts and the infecting viral species. For instance, the human HVs (HHV-1 to HHV-8) are members of *Alpha*-, *Beta*- and *Gamma-herpesvirinae* and have been associated with gingivostomatitis, herpetic keratitis, encephalitis, varicella, mononucleosis, lymphoproliferative malignancy, roseola and sarcoma [2]. The HVs of ruminants belong to the subfamilies *Alpha*- and *Gamma-herpesvirinae*, and infections are associated with rhinotracheitis (ovine HV1, caprine HV1), herpes mammalitis (bovine HV2), meningoencephalitis (bovine HV5), fatal systemic infection (caprine HV1), malignant catarrhal fever (ovine HV2, alcelaphine HV1, 2), ocular disease (cervine HV1) and fatal neurological disorder (bubaline HV1) [4]. The avian and reptilian HVs have so far only been assigned to the subfamily *Alphaherpesvirinae* causing clinical and economic important diseases such as Marek's disease (gallid HV2) and infectious laryngotracheitis (gallid HV1) in poultry, duck plaque enteritis (anatid HV1) in waterfowl, Pacheco's disease (psittacid HV1) in psittacines and fibropapillomatosis (chelonid HV5) in sea turtles [5–10]. Mixed infections of HV species can occur in susceptible hosts leading to a variety of clinical symptoms that may be difficult to diagnose or treat [11–15]. Therefore, there is a need for a sensitive assay that can reliably detect HV species of more than one subfamily in the same clinical samples.

Molecular surveys often employ consensus PCR assays to detect known and novel HVs [16–19]. In fact, several

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new HV species of mammals, reptiles and avians have been discovered using this approach [18, 20–25]. Despite these valuable outcomes, some of the existing consensus PCR assays have variable sensitivity to different HV subfamilies and require a nested PCR format, which can be costly and prone to contamination. Here, we have designed singleplex touchdown consensus PCRs (STC-PCRs) that amplify regions of the DNA polymerase (DPOL) gene of alpha- and gamma-HVs and glycoprotein B (gB) gene of beta-HVs. This non-nested PCR assay was successfully used to detect a wide range of HVs across a broad range of herpesviruses in two independent laboratories.

Materials and methods

Primer design

Degenerate consensus primers were designed for each subfamily based on the alignment of full and partial nucleotide sequences of HVs obtained from GenBank (Supplementary file 1, Table S1). The HV sequences were imported into Geneious 11.1.5 (https://www.geneious.com), and alignments were conducted with ClustalW 2.1 using the default parameters. Primers (Table 1) were manually generated from the conserved regions following visual inspections of the alignments.

DNA preparation and Singleplex Touchdown Consensus PCR

The human and animal HV DNAs tested in this study are shown in Table 2. Viral DNAs were extracted from infected tissues or culture supernatants using the DNeasy Blood and Tissue Kit (Qiagen) as recommended by the manufacturer. Additional DNA extracts were obtained from the Victorian Infectious Diseases Reference Laboratory (VIDRL).

Following assay optimisation (data not shown), the STC-PCR was used to amplify HV DNA in a 20-µL reaction. The reaction mix contained 2 µL of DNA template, 1 µM (beta-HV) or 2 µM (alpha-HV and gamma-HV) primers (Table 1), 200 µM of each deoxynucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.5 U of HotStarTaq polymerase and 1× PCR buffer (Qiagen). The assays were successfully evaluated with ready-to-use pre-mixes including the GoTaq Hot Start Green Master Mix (Promega) and the HotStarTaq Plus master mix (Qiagen) to ensure the assays could be used across a range of PCR chemistries (data not shown). PCR enhancers, including 5% dimethyl sulfoxide (DMSO) and tetramethylammonium chloride (15 mM; TMAC), were also added to the reaction mix. A Touchdown PCR protocol was carried out as outlined in Table 3. The PCR products were analysed on a 1.5% agarose gel made up of 1× TBE buffer and 1× GelRed nucleic acid stain (Biotium).

The specificity of the herpesvirus consensus assays was evaluated by testing a large number of alphaherpesviruses ($n = 22$), betaherpesviruses ($n = 3$) and gammaherpesviruses ($n = 6$). The assay performance was compared to another commonly used herpesvirus nested consensus PCR [19]. The STC-PCR relative sensitivity was tested by assaying a series of 10-fold dilutions of the DNA extracts of representative HVs from each subfamily, and comparing the limit of detection (LOD) with the VanDevanter assay [19]. The assay specificity was also checked by testing the consensus primer pair of one subfamily with the HV DNA templates of other subfamilies. To assess the reproducibility of the assay, herpesviruses were tested using the assays at two independent laboratories, with 22 viruses tested at James Cook University (Townsville, Queensland) and 15 viruses tested at the Australian Centre for Disease Preparedness (Geelong, Victoria).

Results

Overall, a total of 56 primers targeting the conserved regions of different HV genes were designed and tested with a wide range of HV DNAs. Of these, the three primer pairs reported in this study (Table 1; Supplementary file 1, Figure S1) were found to sensitively amplify the DNA sequences of 32 HV species (Table 2). In addition, appropriately sized (specific) single bands were seen (for most of the HVs tested) on

Table 1 List of consensus herpesvirus primers designed for this study

Subfamily	Primer	Sequence (5′ → 3′)	Orientation	Gene	Product length (bp)
Alphaherpesvirinae	AlphaFWD1	AGCATHATYCAGGCBCAYAAY CTSTGYTTYA	Sense	DPOL	265-277
	AlphaREV2	TTRATBGCVRVCTGYTGYTTRTC	Antisense		
Betaherpesvirinae	BetaFWD_gb1	GARGCBTGGTGTHWVGATCA	Sense	gB	564
	BetaREV_gb1	YT[+C]YARR[+T]CRAANACGTT	Antisense		
Gammaherpesvirinae	GammaFWD1	GGVTAYAACRTNKSMAAYTTTGA	Sense	DPOL	650
	GammaREV1	GGRTASAGGCTRGCAAARTC	Antisense		

Table 2 Human and animal herpesviruses tested by STC-PCR and the limit of detection of representative viral species

Subfamily (Genus)	Virus	Results (limit of detection*)		Sample type
		STC-PCR	Nested-PCR [19]	
Alphaherpesvirinae				
Simplexvirus	*Human alphaherpesvirus 1*[a]	+ (10^{-3})	+ (10^{-3})	Human clinical sample
	Human alphaherpesvirus 2[a]	+	+	Human clinical sample
	Macropodid alphaherpesvirus 1[a]	+	+	Cell culture isolate
	Macropodid alphaherpesvirus 2[a]	+	+	Cell culture isolate
	Bovine alphaherpesvirus 2[b]	+	+	Cell culture isolate
Iltovirus	*Gallid alphaherpesvirus 1*[b]	+	+	Cell culture isolate
Mardivirus	*Columbid alphaherpesvirus 1*[a,b]	+ (10^{-4})	+ (10^{-4})	Animal clinical sample; Cell culture isolate
	Meleagrid alphaherpesvirus 1[a,b]	+	+	Animal clinical sample; Cell culture isolate
	Gallid alphaherpesvirus 2[a,b]	+	+	Animal clinical sample; Cell culture isolate
	Anatid alphaherpesvirus 1[b]	+	+	Cell culture isolate
Varicellovirus	*Bovine alphaherpesvirus 1*[a,b]	+ (10^{-5})	+ (10^{-5})	Cell culture isolate
	Human alphaherpesvirus 3[a]	+	+	Human clinical sample
	Equid alphaherpesvirus 1[b]	+	+	Cell culture isolate
	Equid alphaherpesvirus 3[b]	+	+	Cell culture isolate
	Equid alphaherpesvirus 1[b]	+	+	Cell culture isolate
	Felid alphaherpesvirus 1[a,b]	+	+	Cell culture isolate; Vaccine (F3)
Scutavirus	*Chelonid alphaherpesvirus 5*[a]	+	+	Animal clinical sample
Unassigned/unknown	Crocodyline herpesvirus 1[a]	+ (10^{-5})	+ (10^{-5})	Cell culture isolate
	Crocodyline herpesvirus 2[a]	+	+	Cell culture isolate
	Crocodyline herpesvirus 3[a]	+	+	Cell culture isolate
	Phascolarctid herpesvirus[a]	+	+	Animal clinical sample
	Avian herpesvirus	+	+	Cell culture isolate
Betaherpesvirinae				
Cytomegalovirus	*Human betaherpesvirus 5*[a]	+ (10^{-3})	+ (10^{-2})	Human clinical sample
Roseolovirus	*Human betaherpesvirus 6*[a]	+ (10^{-2})	+ (10^{-3})	Human clinical sample
	Human betaherpesvirus 7[a]	+	+	Human clinical sample
Gammaherpesvirinae				
Lymphocryptovirus	*Human gammaherpesvirus 4*[a]	+ (10^{-3})	+ (10^{-1})	Human clinical sample
Manticavirus	*Phascolarctid gammaherpesvirus 1*[a]	+ (10^{-3})	+ (10^{-2})	Animal clinical sample
Rhadinovirus	*Human gammaherpesvirus 8*[a]	+	+	Human clinical sample
	Bovine gammaherpesvirus 4[b]	+	+	Cell culture isolate
Percavirus	*Equid gammaherpesvirus 2*[b]	+	+	Cell culture isolate
Macavirus	*Ovine gammaherpesvirus 2*[b]	+	+	Cell culture isolate

*The limit of the detection was recorded for the representative HVs tested

[a]Viruses tested at James Cook University (JCU) laboratory

[b]Viruses tested at The Australian Centre for Disease Preparedness (AAHL) laboratory

agarose gel following electrophoresis (Fig. 1). The addition of 5% DMSO and 15 mM TMAC greatly improved the sensitivity, specificity, and reproducibility of the PCR reaction (Supplementary file 1, Figure S2).

The detection limits (relative) of the STC-PCR were comparable or lower when compared to the previously reported nested-PCR (Table 2), except for human betaherpesvirus 6, for which the nested PCR detected the viral DNA at one 10-fold dilution lower (Supplementary file 1, Figure S3). The STC-PCR assays produced much 'cleaner' DNA gels than the nested-PCR, which consistently produced many non-specific bands.

Subfamily assay specificity tests showed that the primer pair of one subfamily did occasionally cross-amplify HV DNAs of the other subfamilies (Supplementary file 1, Figure S4). For instance, the alpha-HV primer pair (AlphaFWD1 and AlphaREV2) amplified the DNA of HHV-6 (faint band observed), a member of the subfamily *Betaherpesvirinae*, but did not amplify any gamma-HV DNA (Supplementary file 1, Figure S4). The beta-HV primer pair amplified the DNA of a

Table 3 Optimised touchdown cycling protocol

	Temperature (°C)	Duration	Comments
1. Initial denaturation	95	15 min	Denaturation time vary with master mixes (according to the manufacturers' instruction)
15 cycles of :			
2. Denaturation	94	1 min	
3. Annealing	63 decrement by 1 °C per cycle (− 1 °C/cycle)	1 min	
4. Extension	72	2 min	
35 cycles of:			
5. Denaturation	94	1 min	
6. Annealing	48	1 min	
7. Extension	72	2 min	
1 cycle of:			
8. Final extension	72	10 min	
1 cycle of:			
9. Hold	4	Infinity	

gamma-HV, HHV-4 (faint band), but none of the alpha-HVs. The gamma-HV primers produced varying sized bands for some alpha-HVs including crocodyline HV1 (CrHV-1), meleagridid HV1 (MeHV-1), equine HV4 (EHV-4), bovine HV1 (BoHV-1), HHV-1 and HHV-2 (Supplementary file 1, Figure S4). None of the beta-HV DNA was amplified by the gamma-HV primers (Supplementary file 1, Figure S4).

Discussion

Despite the biological and evolutionary divergence of HVs across the three subfamilies, many evolutionarily conserved core genes still persist [26, 27]. These genes encode proteins that play essential roles in viral entry, nucleic acid synthesis and metabolism, capsid maturation, and virion egress [26]. The DPOL and

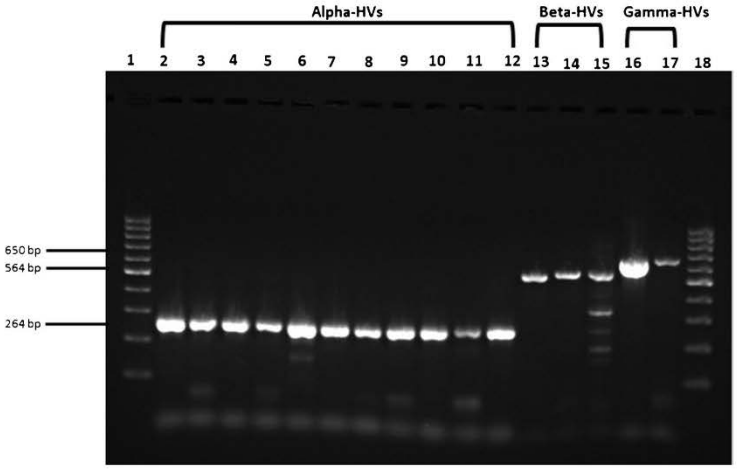

Fig. 1 Electrophoresis of PCR products of HV DNAs obtained by STC-PCR in a 1.5% agarose gel. Lane 1 and 18 contain a 100 bp DNA marker; Lane 2 = *Bovine alphaherpesvirus 1*; lane 3 = *Chelonid alphaherpesvirus 5*, lane 4 = *Macropodid alphaherpesvirus 1*; lane 5 = *Macropodid alphaherpesvirus 2*; lane 6 = *Human alphaherpesvirus 1*; lane 7 = *Human alphaherpesvirus 2*; lane 8 = *Human alphaherpesvirus 3*; lane 9 = *Equid alphaherpesvirus 4*; lane 10 = *Meleagrid alphaherpesvirus 1*; lane 11 = *Gallid alphaherpesvirus 2*; lane 12 = *Felid alphaherpesvirus 1*; lane 13 = *Human betaherpesvirus 5*; lane 14 = *Human betaherpesvirus 6*; lane 15 = *Human betaherpesvirus 7*; lane 16 = *Human gammaherpesvirus 4*; lane 17 = *Human gammaherpesvirus 8*

gB genes are among the most highly conserved genes of HVs and have previously been used as biomarkers for the detection of HVs [18, 19]. In this present study, a singleplex PCR assay targeting conserved genes (DPOL or gB genes) at the subfamily level was developed and successfully used to amplify a broad spectrum of human and animal HV DNAs. Also, the assay produced bright single bands on an electrophoretic gel, which is essential for downstream amplicon sequencing and identification of novel and known HVs.

The addition of 5% DMSO and 15 mM TMAC enhanced the STC-PCR by increasing product yield and ensuring assay reproducibility. High GC content is a common feature of HV genomes [28], and this could pose a challenge during amplification. As previously observed [29, 30], DMSO assists in reducing complex secondary structures and high melting temperature (Tm) associated with GC-rich templates, which in turn reduces duplex stability and allows efficient PCR. TMAC is often recommended when using degenerate primers and helps prevent mispriming by improving stringency of the PCR [31, 32].

In a previous study by VanDevanter et al. [19], a nested PCR using degenerate primers was found to have LODs ranging from a single copy to 100 copies of HV Polymerase DNA per 100 ng of human DNA. Therefore, the sensitivity of the STC-PCR relative to the nested PCR was determined using 10-fold dilutions of representative HVs. The assays were comparable or more sensitive than the nested assay across almost all of the herpesviruses tested. With the improved sensitivity, coupled with cost and time savings, the STC-PCRs can be employed for the epidemiological and clinical detection of known and novel HVs. Some cross-amplification between herpesvirus subfamilies was observed with the STC-PCR due to the high conservation of the targeted DPOL and gB genes at the family level. We consider this cross-amplification a universal feature of the STC-PCR for HV detection; therefore, positive results (amplicons) should be sequenced for onward identification and classification of the detected HVs.

Herpesviruses have been shown to be important pathogens across a large range of vertebrate hosts [1]. Recent initiatives to investigate viral diversity in wildlife hosts have utilised universal PCR assays to discover novel viruses, some with potential clinical and zoonotic concerns [33, 34]. For instance, universal PCR was used to identify six novel herpesviruses in multi-infected samples of chimpanzees (*Pan troglodytes verus*) [35]. Similarly, novel herpesviruses associated with respiratory disease in birds and hepatitis and enteritis in monitor lizards have been detected using universal PCR approaches [36, 37]. Although universal PCR assays have been an invaluable tool for these viral discovery initiatives, many of these assays can be problematic due to poor sensitivity, low specificity and contamination issues (especially with nested assays). Here, we have designed and evaluated novel singleplex universal PCR assays that will be useful for detection of known and novel herpesviruses from human and animal clinical samples.

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Data Availability All data analysed during this study are included in this published article [Supplementary file 1].

Code Availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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GENOME SEQUENCES

Draft Genome Sequence of a Novel Adenovirus Recovered from the Metagenome of Agile Wallabies

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ABSTRACT Here, we report the draft genome sequence of a novel agile wallaby adenovirus that was detected in the fecal metagenome of agile wallabies. The genome is 31,512 bp long, with a G+C content of 34.4%. Currently, the pathogenic and zoonotic potential of this novel virus is unknown.

Adenoviruses are nonenveloped, icosahedral viruses with linear, unsegmented double-stranded DNA genomes (1). The genomes of adenoviruses range in size from 26 to 48 kb and commonly contain between 22 and 40 genes (1, 2). The family *Adenoviridae* is divided into six genera, namely, *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, *Ichtadenovirus*, and *Testadenovirus* (1, 3). Adenoviral infections do not always result in disease, although they have been associated with both single and multipathogen disease processes (4–8).

The novel Agile wallaby atadenovirus 1 (AwAdV-1) described in this report belongs to the genus *Atadenovirus* and was originally identified in the metagenome of free-ranging agile wallabies (*Notamacropus agilis*) (G. R. Okoh, E. Ariel, D. Whitmore, P. F. Horwood, submitted for publication). Briefly, five fresh fecal samples were collected from the ground at grazing sites around James Cook University and Townsville University Hospital (Townsville, Australia) in 2021. The samples were homogenized, pooled, and then virally enriched by filtration (0.25 μm), ultracentrifugation (100,000 $\times$ *g*), and digestion with DNase I (20 U/mL). Viral DNA was then extracted using QIAamp MinElute virus kit (Qiagen). Library preparation using the Nextera DNA XT kit and Illumina sequencing (NovaSeq 6000) were performed at Macrogen (Seoul, South Korea) in paired-end 151-bp format. For this report, the sequencing reads (71,170,820) were trimmed (Trimmomatic v0.39) (9) to remove low-quality reads, normalized using BBNorm v39.01 (https://sourceforge.net/projects/bbmap/), and *de novo* assembled using SPAdes v3.15.5 in "careful" mode (10). The resulting contigs were searched using Diamond BLASTX (11) against the NCBI nonredundant (nr) protein database to identify the contigs corresponding to adenovirus. To assemble the genome, the reads were mapped to the identified adenoviral contig using Geneious v11.1.5 (https://www.geneious.com). Prediction of open reading frames (ORFs) was performed using Glimmer3 in Geneious v11.1.5, and ORF annotations were determined by conducting a BLASTX search against the NCBI nr protein databases (12). All tools were run with default parameters unless otherwise specified.

The assembled genome of AwAdV-1 was found to be 31,512 bp long, with a coverage depth of 22$\times$ and 34.4% G+C content. The genome was predicted to contain 32 ORFs with an orientation typical of atadenoviruses. Of the 32 ORFs, 26 were annotated as having various similarities to the coding genes of other atadenoviruses (Tables 1). The IVa2, penton base protein, PX, and hexon genes showed the highest amino acid identity (71% to 87%) to the reference mammalian atadenoviruses (Table 1). The AwAdV-1 genome possesses multiple insertions and deletions in most of the genes except IVa2, pX, pVI, pVIII, and U-exon. Two fiber genes, namely, fiber and IV-1 (homologous to the fiber 2 gene in lizard adenovirus 2), were present in the genome of AWAdV-1, instead of the single long fiber gene in mammalian atadenoviruses. Phylogenetic analysis based on the full amino

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The authors declare no conflict of interest.

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TABLE 1 Sequence comparison with the genomes of mammalian atadenoviruses

ORF	Description (putative)	Data for virus (GenBank accession no.):[a] Agile wallaby atadenovirus 1 Length (nt)	Length (aa)	*Ovine adenovirus* (NC_004037) Length (nt)	Length (aa)	Amino acid identity (%)	*Bovine adenovirus D* (NC_002685) Length (nt)	Length (aa)	Amino acid identity (%)	*Bovine adenovirus E* (NC_020074) Length (nt)	Length (aa)	Amino acid identity (%)	*Odocoileus adenovirus 1* (NC_035619) Length (nt)	Length (aa)	Amino acid identity (%)
orf0001	Hypothetical protein	255	85	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf0002	Hypothetical protein	402	134	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00003	p32K	1,032	344	861	286	39	819	272	45	891	296	40	930	309	35
orf00004	LH1	396	132	363	120	29	378	125	29	378	125	26	393	130	25
orf00005	E1B 55K	1,293	431	1149	382	43	1,161	386	42	1,152	383	45	1,146	381	43
orf00006	IVa2	903	301	984	327	75	966	321	74	966	321	72	1,209	402	72
orf00007	Pol	3,243	1,081	3,216	1,071	58	3,222	1,073	58	3,222	1,073	57	3,228	1,075	58
orf00008	pTP	1,773	591	1,788	595	50	1,803	600	51	1,803	600	51	1,800	599	51
orf00009	52K	987	329	1,008	335	62	1,059	343	62	1,035	344	60	1,014	337	58
orf00010	pIIIa	1,767	589	1,707	568	53	1,722	573	54	1,551	516	54	1,749	582	53
orf00011	Penton base protein	1,347	449	1,359	452	68	1,353	450	67	1,359	452	68	1,353	450	71
orf00012	pVII	345	115	336	111	54	360	119	57	357	118	56	354	117	51
orf00013	pX	102	34	216	71	87	216	71	81	219	72	0	No data	No data	No data
orf00014	pVI	669	223	666	221	54	603	200	57	612	203	56	678	225	54
orf00015	Hexon	2,730	910	2,736	911	74	2,733	910	72	2,733	910	76	2,733	910	74
orf00016	23K endoprotease	606	202	606	201	59	606	201	60	606	201	61	606	201	60
orf00018	DNA binding protein	999	333	1,149	382	56	1,143	380	58	1,140	379	56	1,158	385	57
orf00020	100K	2,055	685	1,878	625	54	1,887	628	56	1,887	628	54	1,914	637	55
orf00021	33k	512	170	402	133	39	405	134	65	408	135	39	414	137	38
orf00022	pVIII	780	260	654	217	44	669	222	44	672	223	45	681	226	49
Orf00023	U-exon	165	55	177	58	35	165	54	54	165	54	54	165	54	54
orf00024	Fiber	891	297	1,632	543	36	1,608	535	36	1,332	443	43	1,422	473	29
orf00025	IV-1	1,554	518	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00026	E4.3	600	200	714	237	34	654	217	38	657	218	38	705	234	33
orf00027	E4.2	678	226	663	220	40	678	219	42	660	219	42	678	219	39
orf00028	E4.1	441	147	429	142	36	429	142	37	429	142	38	429	142	30
orf00029	RH0	381	127	No data	No data	No data	564	187	45	No data	No data	No data	No data	No data	No data
orf00030	RH5	606	202	597	198	26	624	207	27	651	216	24	624	207	34
orf00031	Hypothetical protein	372	124	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00032	Hypothetical protein	201	67	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00033	Hypothetical protein	366	122	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
orf00034	Hypothetical protein	372	124	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data

[a] aa, amino acids.

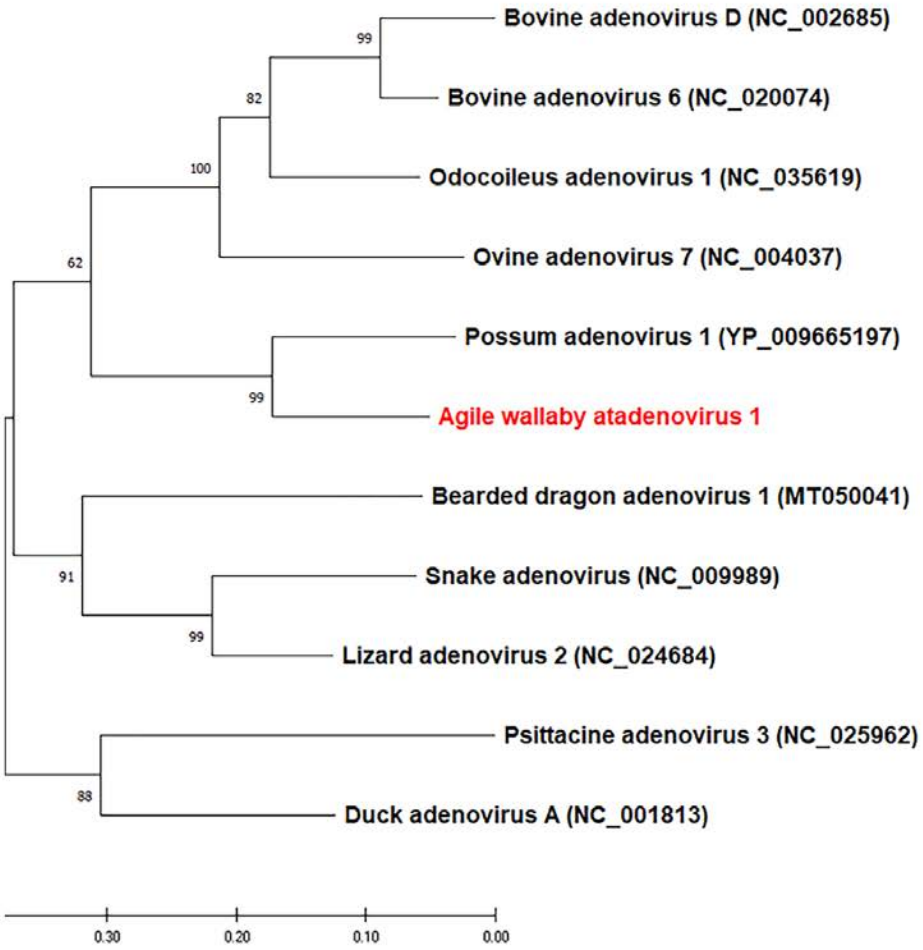

FIG 1 Phylogenetic analysis of Agile wallaby atadenovirus 1 (shown in red), based on the amino acid sequence of penton-based protein. All the sequences used in this analysis belong to the genus *Atadenovirus*. Following multiple sequence alignment of amino acid sequences using the Muscle program in Geneious v11.1.5, a maximum likelihood phylogenetic tree was constructed using MEGA X (13) with the best model of amino acid substitution (LG + G) and 1,000 bootstrap replications.

acid sequence of penton base protein showed that AwAdV-1 belongs to the genus *Atadenovirus* and forms a distinct cluster with another marsupial adenovirus known as possum adenovirus 1 (Fig. 1).

The pathogenic potential of AwAdV-1 is unclear; however, it could be a suitable candidate for future research in vaccinology, diagnostics, and therapeutics.

Data availability. The raw sequence reads for this study have been deposited in the NCBI SRA database under BioProject accession number PRJNA907146 and BioSample accession number SAMN31952915. The novel genome sequence has been deposited at GenBank under the accession number OQ792214.

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