



Murdoch
UNIVERSITY

MURDOCH RESEARCH REPOSITORY

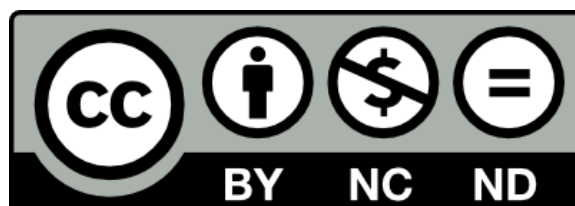
This is the author's final version of the work, as accepted for publication following peer review but without the publisher's layout or pagination.

The definitive version is available at

<http://dx.doi.org/10.1016/j.vetmic.2015.09.013>

Hyndman, T.H., Shilton, C.M., Wellehan, J.F.X., Davis, S., Isberg, S.R., Phalen, D. and Melville, L. (2015) Molecular identification of three novel herpesviruses found in Australian farmed saltwater crocodiles (*Crocodylus porosus*) and Australian captive freshwater crocodiles (*Crocodylus johnstoni*). *Veterinary Microbiology*, 181 (3-4). pp. 183-189.

<http://researchrepository.murdoch.edu.au/29342/>



Accepted Manuscript

Title: Molecular identification of three novel herpesviruses found in Australian farmed saltwater crocodiles (*Crocodylus porosus*) and Australian captive freshwater crocodiles (*Crocodylus johnstoni*)

Author: Timothy H. Hyndman Cathy M. Shilton James F.X. Wellehan Jr Steven Davis Sally R. Isberg David Phalen Lorna Melville

PII: S0378-1135(15)30031-6
DOI: <http://dx.doi.org/doi:10.1016/j.vetmic.2015.09.013>
Reference: VETMIC 7096

To appear in: *VETMIC*

Received date: 4-5-2015
Revised date: 4-9-2015
Accepted date: 16-9-2015

Please cite this article as: Hyndman, Timothy H., Shilton, Cathy M., Wellehan, James F.X., Davis, Steven, Isberg, Sally R., Phalen, David, Melville, Lorna, Molecular identification of three novel herpesviruses found in Australian farmed saltwater crocodiles (*Crocodylus porosus*) and Australian captive freshwater crocodiles (*Crocodylus johnstoni*). *Veterinary Microbiology* <http://dx.doi.org/10.1016/j.vetmic.2015.09.013>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Molecular identification of three novel herpesviruses found in Australian farmed saltwater crocodiles (*Crocodylus porosus*) and Australian captive freshwater crocodiles (*Crocodylus johnstoni*)

Timothy H Hyndman^{a*} t.hyndman@murdoch.edu.au, Cathy M Shilton^b cathy.shilton@nt.gov.au, James FX Wellehan Jr.^c wellehanj@uf.edu, Steven Davis^b steven.davis@nt.gov.au, Sally R Isberg^{d,e} sally@crocresearch.com.au, David Phalen^d david.phalen@sydney.edu.au, Lorna Melville^b lorna.melville@nt.gov.au

^aMurdoch University, School of Veterinary and Life Sciences, Murdoch, Western Australia, 6150, Australia

^bBerrimah Veterinary Laboratories, Northern Territory Government, GPO Box 3000, Darwin, Northern Territory, 0801, Australia

^cDepartment of Small Animal Clinical Sciences, University of Florida College of Veterinary Medicine, Gainesville, Florida, 32610, USA

^dThe University of Sydney, Sydney, New South Wales, 2006, Australia

^eCentre for Crocodile Research, Noonamah, Northern Territory, 0837, Australia

* Corresponding author. Tel.: +61 08 9360 7348.

Highlights

- Crocodiles are farmed in northern Australia for skin which is used for leather
- Four debilitating disease syndromes have been described in them
- Herpesviruses were isolated from affected crocodiles
- Three novel herpesviruses were identified from isolates by PCR and phylogeny
- The role that these herpesviruses play in disease needs further investigation

Abstract

As part of a larger investigation into three emerging disease syndromes highlighted by conjunctivitis and pharyngitis, systemic lymphoid proliferation and encephalitis, and lymphonodular skin infiltrates in farmed saltwater crocodiles (*Crocodylus porosus*) and one emerging syndrome of systemic lymphoid proliferation in captive freshwater crocodiles (*C. johnstonii*), cytopathic effects (CPE), including syncytial cell formation, were observed in primary crocodile cell lines exposed to clarified tissue homogenates from affected crocodiles. Ten cell cultures with CPE were then screened for herpesviruses using two broadly-reactive herpesvirus PCRs. Amplicons were obtained from 9 of 10 cell cultures and were sequenced. Three novel herpesviruses were discovered and the phylogenetic analysis of these viruses showed there was a 63% Bayesian posterior probability value supporting these viruses clustering with the subfamily *Alphaherpesvirinae*, and 100% posterior probability of clustering with a clade containing the *Alphaherpesvirinae* and other unassigned reptile herpesviruses. It is proposed that they are named Crocodyline herpesvirus (CrHV) 1, 2 and 3. CrHV1 and 2 were only isolated from saltwater crocodiles and CrHV3 was only isolated from freshwater crocodiles. A duplex PCR was designed that was able to detect these herpesviruses in formalin-fixed paraffin-embedded tissues, a sample type that neither of the broadly-reactive PCRs was able to detect these herpesviruses in. This work describes the isolation, molecular detection and phylogeny of these novel herpesviruses but the association that they have with the emerging disease syndromes requires further investigation.

Keywords: Reptile; Alphaherpesvirus; Virus isolation; Phylogeny; PCR.

1. Introduction

Herpesviruses are enveloped viruses that have a single, linear, double-stranded DNA genome. The order *Herpesvirales* is divided into three families: *Malacoherpesviridae*, found in molluscs, *Alloherpesviridae*, the herpesviruses of ray-finned fish and amphibians, and *Herpesviridae*, found in mammals, birds and reptiles (Pellet et al., 2012). To date, all known reptilian herpesviruses are either within the *Alphaherpesvirinae*, or phylogenetic analyses have found they are basal to, but most closely related to, the currently accepted alphaherpesviruses (McGeoch and Gatherer, 2005; Jungwirth et al., 2014). As large DNA viruses with intranuclear replication, herpesviruses generally have very high host fidelity and often appear to have co-diverged over their course of evolution along with their hosts (Pellet and Roizman 2007).

In reptiles, herpesviruses, or herpesvirus-like particles detected by electron microscopy, have been described in a range of lizard, snake, and chelonian species with and without observable signs of disease (reviewed by Jacobson, 2007 and Marschang, 2011). Where disease was noted, a wide range of signs and pathological processes have been detected including stomatitis, rhinitis, conjunctivitis, tracheitis, oesophagitis, hepatitis and fibropapillomas (Jacobson et al., 1986; Origi, 2006; Wellehan et al., 2004).

Herpesviruses in crocodylians have only been described in two investigations. In the first, herpesvirus-like particles were seen in degenerate superficial epidermal cells in a six-month old saltwater crocodile (*Crocodylus porosus*) that had been farm-hatched in the Northern Territory of Australia and then transported to a facility in Victoria, Australia for stress research (McCowan et al., 2004). In the second investigation, a novel herpesvirus, named Crocodylid herpesvirus 1, was reportedly detected by sequencing of a PCR product from cloacal lesions of juvenile alligators (*Alligator mississippiensis*) from two alligator farms in the USA (Govett et al., 2005). However,

since publication, sequence 100% homologous to this was found in several Greek (*Testudo graeca*) and Hermann's tortoises (*T. hermanni*) (Marschang et al., 2006). The partial DNA polymerase sequence from the alligator report was later revised in GenBank as "Tortoise herpesvirus Allmi1" and the authors suggested on GenBank that this herpesvirus was a probable contaminant (GenBank Accession Number: AY913769.1).

In Australia, crocodiles are intensively farmed to produce skins for the luxury leather market. In farmed saltwater (*Crocodylus porosus*) and captive freshwater (*C. johnstoni*) crocodiles in the Darwin region of the Northern Territory of Australia, four disease syndromes have recently emerged; three in saltwater crocodiles and one in freshwater crocodiles. This study describes the isolation, molecular identification and phylogenetic placement of three novel herpesviruses isolated from crocodiles with these syndromes and PCR tests that can be used to detect them.

2. Materials and Methods

2.1 Crocodile farms and animals

Samples in this study originated from two large crocodile farms, Farms 1 and 2, within 60 km of Darwin in the Northern Territory. Conjunctivitis and pharyngitis (CP) occurred in saltwater crocodiles on both farms, while systemic lymphoid proliferation and encephalitis (SLPE) and lymphonodular skin infiltrates (LNS) occurred only on Farm 2. In addition to saltwater crocodiles, Farm 1 also raised small numbers of freshwater crocodiles in which freshwater crocodile systemic lymphoid proliferation (FSLP) was recognised. All crocodiles originated from eggs that were either collected from nests in the wild or were laid on the farms from captive breeding stock.

All samples in this study originated from crocodiles from these two farms and had been submitted to Berrimah Veterinary Laboratories for post-mortem examination between 2006 and 2010. The examined carcasses were from either recently deceased or euthanased animals. A full selection of tissue samples were collected for histological assessment (manuscript in preparation). These tissue samples were placed into 10% formalin prior to paraffin embedding. In addition, conjunctival and pharyngeal swabs, and samples of skin, liver, brain, spleen, kidney, lung and thymus were stored at -20 °C until used for virus isolation.

2.2 Cell line development and virus isolation

Tissues for cell line development were aseptically removed from hatchling crocodiles less than 24 hours old. Segments of kidney, liver, trachea, heart, lung and subcutaneous tissue were individually placed into Medium 199 (M199) tissue culture medium (catalogue number 31100-019, GIBCO, Life Technologies, Grand Island, USA) supplemented with 15% bovine foetal calf serum, 500 unit/mL penicillin, 0.5 mg/mL streptomycin and 1.25 µg/mL amphotericin B and incubated at room temperature for two hours. The tissue was then removed, finely macerated to a paste, resuspended in 2 mL of unsupplemented M199 and then 2 mL of 0.12% trypsin solution was added before the mixture was incubated with stirring for 10 minutes at room temperature. The cell suspension was then resuspended in 10 mL of M199 supplemented with 15% foetal bovine serum, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B and 20 ng/mL of epidermal growth factor (catalogue number E4127, Sigma-Aldrich, St.Louis, USA), filtered through a fine mesh screen and dispensed into 25 cm² tissue culture flasks and incubated at 28 °C. The flasks were examined every two days and when any flask exhibited cell monolayer growth exceeding 70% of the growth surface, the cells were then passaged to new

flasks using standard trypsinisation procedures. After five passages, the epidermal growth factor was removed from the M199 culture media.

For the isolation of viruses displaying cytopathic effects (CPE), two cell lines were used for each virus isolation attempt: one derived from kidney, in which the cultured cells had morphology suggestive of epithelial cells, and one derived from liver, subcutaneous connective tissue, heart or trachea, in which the cells had morphology suggestive of fibroblasts. Tissue samples and particulate matter from swabs were placed into 5 mL of brain-heart infusion broth that was supplemented with penicillin G, streptomycin and amphotericin B. Using a mortar and pestle, samples were then homogenised. Following clarification at 670 g for 10 min, the supernatant was filtered through a 0.45 µm filter and 0.3 mL of the filtered supernatant was then inoculated onto a monolayer of >70% confluent primary cells in each of two 25 cm² flasks. The cells were examined for CPE every three days for 21 d and then following passage, were again examined every three days for another 21 d. Passaging was accomplished by sonicating the flask for 20 min, then vigorously shaking the flask and transferring 0.3 mL of the mixture onto a >70% confluent monolayer. Cultures showing CPE, typified by loss of confluence of the cell monolayer, cell rounding and syncytial cell formation (Figure 1), were harvested and stored at -70 °C for later analysis for virus identification. If no CPE was observed at any time, further testing was not pursued and the culture was considered negative for CPE-displaying viruses.

2.3 Herpesvirus PCR

The samples tested by PCR are listed in Table 1. Ten viral isolates and four sets of formalin-fixed paraffin-embedded (FFPE) tissue were selected that collectively represented both crocodile host species, both farms and all four disease syndromes. Fresh frozen tissue was unavailable for PCR testing. DNA was extracted from a 200 µL aliquot of frozen-thawed cell

culture homogenate using the MagMAX™ Viral RNA Isolation Kit (Ambion, Austin, Texas) according to the manufacturer's instructions. For FFPE tissues, samples were processed using the MELT™ Total Nucleic Acid Isolation System (Cat. No. AM1983, Ambion, Austin, Texas) according to the manufacturer's instructions except for a minor modification. Samples were first deparaffinised in two washes of xylene and then the xylene was cleared with two washes of ethanol. Next, deparaffinised tissues were digested overnight at 50 °C in the digestion cocktail provided with the Ambion kit (Abramovitz et al. 2008). DNA was always eluted into 30 µL of elution buffer. All the PCRs used in this study targeted the conserved DNA-dependent DNA polymerase gene of herpesviruses and are listed in Table 2. Initially, a pan-*Herpesviridae* nested PCR was used as previously described (VanDevanter et al. 1996). *Equine herpesvirus 1* served as a positive control while uninfected crocodile cell culture homogenate from two separate culture flasks that had not been inoculated with crocodile tissue or swabs, served as negative controls.

PCR products were separated using 2% (w/v) agarose gel electrophoresis and visualised using 0.005 % SYBR safe (v/v; Invitrogen, Mulgrave, Victoria) and a transilluminator (DR88M Dark Reader non-UV Transilluminator; Clare Chemical Research Inc., Dolores, Colorado). Bands of appropriate size were excised from the gel using a sterile scalpel blade, were purified using the Purelink Quick Gel Extraction Kit (Invitrogen, Mulgrave, Victoria) and then directly sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, California). Primer sequences were excluded from amplicons that were then compared to the GenBank database (www.ncbi.nlm.nih.gov/Genbank/index.html).

These short sequences (169 and 181 nucleotides) were then used to design alternative PCR primers to detect the crocodile herpesviruses discussed in this paper. Using standard

settings in Geneious (version 6.1.7, Biomatters), a number of primers were suggested. These primers were then tested for specificity *in silico* using BLASTn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Only primers with BLASTn results that produced E-values of greater than 35 for currently known herpesviruses were considered. The primer sequences chosen and their PCR product sizes are listed in Table 2. For this PCR, DNA from uninfected primary crocodile cell lines, *Equine herpesvirus 1*, *Feline herpesvirus 1* (Feligen RCP vaccine, Virbac, Australia) and *Gallid herpesvirus 2* (HVT vaccine, Bioproperties, Australia) served as negative controls. To partially optimise this PCR, two different final concentrations of magnesium were trialled (1.5 mM and 3 mM) at three different annealing temperatures (47 °C, 50 °C and 53 °C). The partially optimised conditions were selected based on band intensity and the absence of spurious bands (data not shown). Ultimately, 1 µL of extracted DNA was added to 1 µM (final concentration) of each of the four primers (Crocodyline herpesviruses 1 and 2/3, see Table 2) and the final volume was made up to 20 µL using Platinum® Blue PCR Supermix (Cat. No. 12580-015, Invitrogen, Victoria, Australia). No additional magnesium was added to the PCR mastermix (approximately 1.5 mM final concentration). Cycling conditions consisted of 94 °C x 2 m, 40 x (94 °C x 20 s, 53 °C x 45 s, 72 °C x 45 s). Sequencing of appropriately sized amplicons was performed as above.

To exceed the minimum requirement of 200 nucleotides for GenBank submission, testing using pan-*Herpesvirales* PCR primers was completed as described elsewhere (Hanson et al. 2006). The sequence from the duplex PCR (detecting Crocodyline herpesviruses 1, 2 and 3) lies entirely within the sequence derived from the second round of the nested PCR described by VanDevanter et al. (1996), which in turn fits entirely within the sequence from the PCR described by Hanson et al. (2006). These longer sequences (472 - 484 nucleotides) were used for

phylogenetic analyses. The primers designed for the specific detection of the crocodile herpesviruses were then re-examined against the additional sequence information obtained. Using the same process mentioned above, the same four primers were identified as being the most suitable (data not shown).

2.4 Phylogeny

Predicted homologous 153-167 amino acid sequences of DNA-dependent-DNA polymerase were aligned using MAFFT (Kato and Toh, 2008). Partial homologous amino acid sequences for which full-length sequence was not available were included, with ambiguities added for unknown amino acids, for Gerrhosaurid herpesvirus 1 (59 amino acids), Gerrhosaurid herpesvirus 2 (59 amino acids), Gerrhosaurid herpesvirus 3 (60 amino acids), Varanid herpesvirus 1 (58 amino acids), Lacertid herpesvirus 1 (59 amino acids), Tortoise herpesvirus 1 (60 amino acids), Tortoise herpesvirus 2 (60 amino acids), Tortoise herpesvirus 4 (141 amino acids), Indotestudo herpesvirus (60 amino acids), Cooter herpesvirus (60 amino acids), and Red-eared slider herpesvirus (60 amino acids). Indotestudo herpesvirus, Cooter herpesvirus, and Red-eared slider herpesvirus are not in GenBank due to the recent requirement of a minimum of 200 nucleotides for submission, but amino acid sequences may be seen in the supplemental alignment figure, and nucleotide sequences are available on request. Lacertid herpesvirus 1 (GenBank accession no. ACD64983) was designated as the outgroup. Bayesian analyses of amino acid alignments were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) on the CIPRES server (Miller et al., 2010), with gamma distributed rate variation and a proportion of invariant sites, and mixed amino acid substitution models. The first 25% of 1,000,000 iterations were discarded as a burn in.

Maximum likelihood (ML) analyses of each alignment were performed using RAxML on the CIPRES server (Stamatakis et al, 2008), with gamma distributed rate variation and a proportion of invariant sites. The amino acid substitution model with the highest posterior probability in the Bayesian analysis was selected. Bootstrap analysis was used to test the strength of the tree topology, with 1000 subsets (Felsenstein, 1985).

3. Results

Cell lines were successfully propagated from kidney, liver, trachea, heart, lung and subcutaneous tissue samples. The results of PCR testing are summarised in Table 1. Herpesviruses were detected in nine of ten cell cultures demonstrating CPE. The crocodile herpesvirus PCR was able to detect the crocodile herpesviruses in FFPE tissue (n = 4) whereas the two broadly-reactive herpesvirus PCRs (VanDevanter et al., 1996; Hanson et al., 2006) did not. Of note, herpesvirus was detected in a combined sample of FFPE liver, kidney, spleen and fat body but a herpesvirus could not be detected by PCR in an isolate with CPE that originated from the lung of this same crocodile. In our hands, the Hanson et al. (2006) PCR detected herpesviruses in more of the tested isolates (9/10) than the PCR described by VanDevanter et al. (1996) (7/10).

A Bayesian tree is shown in Figure 2. Bayesian phylogenetic analysis found that the WAG model of amino acid substitution was most probable with a posterior probability of 1.000 (Whelan and Goldman, 2001). Based on phylogenetic analysis and nucleotide and amino acid identities (Table 3), three novel and distinct herpesviruses were discovered. Fitting with herpesvirus naming conventions (Pellet et al., 2012), we propose that these three viruses are named Crocodyline herpesvirus 1 (CrHV1), Crocodyline herpesvirus 2 (CrHV2) and

Crocodyline herpesvirus 3 (CrHV3). While the genetic distance seen between CrHV2 and 3 is smaller than the distance between *Human herpesvirus 1* and *Human herpesvirus 2*, which are often used as a benchmark for distinguishing species (Pellet et al., 2012), the ecological distinction of host species (CrHV2 was only found in salt water crocodiles and CrHV3 was only found in freshwater crocodiles) suggests that CrHV2 and CrHV3 are separate species. There was a 63% Bayesian posterior probability value supporting these viruses clustering with the subfamily *Alphaherpesvirinae*, and an 80% posterior probability supporting CrHV2 and CrHV3 clustering with *Mardivirus*, *Simplexvirus* and *Varicellovirus*. The *Betaherpesvirinae* and *Gammaherpesvirinae* clustered separately from the *Alphaherpesvirinae* and unclassified reptile herpesviruses, with 100% posterior probability. The partial DNA-dependent DNA polymerase sequences from CrHV1, CrHV2 and CrHV3 have been uploaded into GenBank under the accession codes KP313603, KP313604 and KP313605 respectively.

Sequencing of PCR products revealed that CrHV1 and CrHV2 both occurred in saltwater crocodiles whereas CrHV3 occurred only in the two freshwater crocodiles with FSLP (Table 1). CrHV1 and CrHV2 were detected in separate CP cases and at both farms, while CrHV2 was detected in the three cases of SLPE and one case of LNS investigated from Farm 2 (Table 1).

4. Discussion

In this study, a number of herpesviruses from Australian crocodiles were isolated and phylogenetically analysed. Based on the relatedness of 472-484 nucleotides of the conserved DNA polymerase gene from each herpesvirus, coupled with differences in host species, it is our opinion that the herpesviruses described in this study represent three novel and distinct viruses. In the previous work by Govett et al. (2005), a new crocodylian herpesvirus was announced and

tentatively named Crocodylid herpesvirus 1 but, as mentioned above, the 100% sequence homology with a tortoise herpesvirus suggests that this finding appears to be an error. There is precedent set for use of host genus rather than family name when naming herpesviruses; several primate viruses infecting the genus *Macaca* have been renamed as Macacine herpesvirus. To help prevent confusion between the herpesviruses in our paper and the one described by Govett et al. (2005), we propose use of the genus (crocodyline), instead of family (crocodylid), in naming these viruses. Should a herpesvirus emerge with strong evidence that its host is an alligator, we would suggest that the name Alligatorine herpesvirus be considered (after its genus).

This data set used in the alignment has a number of sequences that are partial. The confidence measurement typically used with maximum likelihood analyses, bootstrapping, does not actually measure how likely it is that a clade clusters together, but rather how often subsets of the data agree on a given clade. Thus it is a measurement of internal agreement rather than probability (Susko, 2009). Inclusion of a number of sequences that are less than half the length of the alignment will artifactually depress some bootstrap values. The partial sequences included are all reptile sequences, and significantly improve our taxon sampling in a crucial region of the tree. Improved taxon sampling significantly increases accuracy of phylogenetic analyses (Havird and Miyamoto, 2010; Flynn et al, 2005). This should be taken into account when interpreting our ML bootstrap values.

Our phylogenetic analysis shows weak support that these crocodyline herpesviruses cluster with the known herpesviruses of the other extant archosaur (birds and crocodylians) clade, the dinosauria (birds), as well as those of the turtles and the mammalian alphaherpesviruses. With the exception of the mammalian alphaherpesviruses, the viruses of the turtle/archosaur

hosts otherwise may form a monophyletic group. The branch lengths of the mammalian alphaherpesviruses are fairly short compared to the more diverse clade infecting mammals, the betaherpesviruses and gammaherpesviruses. The mammalian alphaherpesviruses may represent a host jump from an archosaur.

An unexpected finding of our investigation was that the two most closely related crocodyline herpesviruses, CrHV2 and CrHV3, came from different hosts (saltwater and freshwater crocodiles respectively), yet it was in a single host, the saltwater crocodile, that two divergent viruses were found (CrHV1 and CrHV2). It is unknown what the explanation for this is but a simple explanation is that crocodyline herpesviruses from the clade that CrHV2 and CrHV3 cluster within can be found in both saltwater and freshwater crocodiles, but herpesviruses belonging to the clade that contains CrHV1 are only found in saltwater crocodiles. It is also possible that CrHV1 (or a closely related herpesvirus) will eventually be detected in freshwater crocodiles. Another explanation is that “host jumping” of CrHV3 from a freshwater crocodile to a saltwater crocodile lead to the evolution of CrHV2, or similarly, CrHV2 “jumped host” from saltwater crocodiles into freshwater crocodiles and then evolved into CrHV3.

In the current study, we found that our duplex PCR was able to detect CrHV in FFPE tissues whereas the broadly-reactive herpesvirus PCRs described by Hanson et al. (2006) and VanDevanter et al. (1996) were not. The duplex PCR detects a shorter segment of CrHV DNA than both of the broadly-reactive PCRs (108-113 nucleotides compared to 222-542 nucleotides) and so may be more suitable for the degraded DNA in FFPE samples.

We were able to detect herpesvirus DNA in aliquots from all inoculated cell cultures, except one. It is possible that this culture contained a herpesvirus that could not be amplified

with the primer sets we used. Alternately, it is possible that another virus capable of causing similar CPE was present in it.

It was not the purpose of this report to create a strong aetiological or epizootiological link between CrHV1, CrHV2, and CrHV3 and the four syndromes occurring in captive crocodiles in the Northern Territory of Australia. Instead, the purpose was to characterise these viruses and then to investigate PCR testing that could detect CrHV1, 2 and 3. By doing this, we provide future investigators with the tools necessary to screen virus isolates, formalin-fixed tissues, necropsy specimens and possibly live animals for currently known CrHVs. Given that additional novel herpesviruses may be found in Australian crocodiles and would seem likely to be present in other crocodylian species globally, we recommend that broadly-reactive PCRs are not abandoned in favour of the duplex PCR described in this manuscript. Instead, both PCRs should be considered. It is possible, perhaps likely, that investigation into these disease syndromes will be complicated by the presence of other pathogens and therefore, unbiased molecular techniques, such as next-generation sequencing, should be considered as well.

In summary, this study reveals three novel herpesviruses detected in Australian crocodiles with emerging disease syndromes. To investigate the causality between these herpesviruses and the four described syndromes, several lines of investigation could be pursued. Collation of herpesvirus test results from detailed diagnostic investigations on large numbers of crocodiles with the syndromes, including comparison to crocodiles without the syndromes, would enable judgement of the plausibility that these syndromes are *caused* by herpesviruses. Demonstrating the temporal and spatial association of viral antigen and/or nucleic acid with lesions by immunohistochemistry or *in situ* hybridisation, and experimentally reproducing the

disease syndromes in transmission studies are also important to further our understanding of these disease syndromes.

5. Conclusion

A number of disease syndromes have recently been described in northern Australian captive crocodiles. Consequently, there are both animal welfare and financial reasons to investigate the cause of these syndromes. A selection of viruses representing the two host species, two affected farms and all four described syndromes, were analysed by molecular methods for the presence of herpesviruses. Ultimately, three novel herpesviruses were found that have been named Crocodyline herpesvirus 1, 2 and 3. There is strong phylogenetic evidence that these viruses cluster with the alphaherpesviruses separately from the betaherpesviruses and gammaherpesviruses. However, additional studies using longer sequences and sequences from multiple genes will be needed to define their true relationships with other avian, reptilian, and mammalian herpesviruses. We also report on the development of PCR testing that can be used to detect these viruses. These tests have the potential to be used in studying the epizootiology of these viruses.

6. Acknowledgements

This research was funded in part by the Australian Government Rural Industries Research and Development Corporation (RIRDC). Some of the results of this research were briefly summarised in a non-peer reviewed final report for the funding body: Melville L., Davis S., Shilton C., Isberg, S., Chong, A., Gongora, J. 2012. Hunting for viruses in crocodiles: viral and endogenous retroviral detection and characterization in farmed crocodiles. Barton, Australia:

Australian Government Rural Industries Research and Development Corporation Publication No. 12/011.

We would like to thank Professor Graham Wilcox for providing us with equine herpesvirus to serve as a positive control for some of our experiments.

References

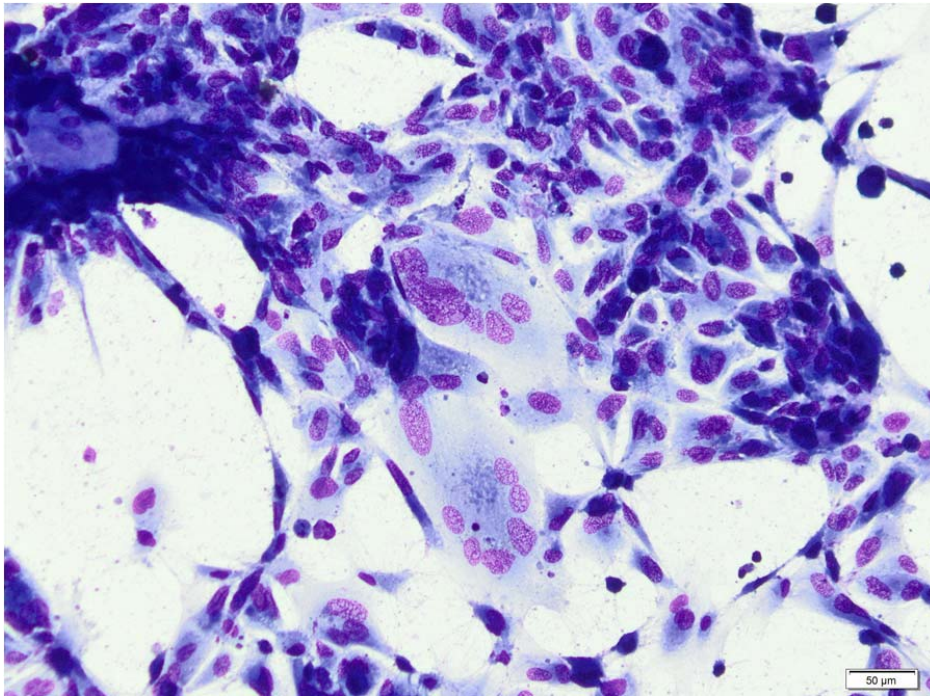
- Abramovitz, M., Ordanic-Kodani, M., Wang, Y., Li, Z., Catzavelos, C., Bouzyk, M., Sledge, G.W. Jr., Moreno, C.S., Leyland-Jones, B. 2008. Optimization of RNA extraction from FFPE tissues for expression profiling in the DASL assay. *Biotechniques* 44(3), 417-423.
- Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.
- Flynn JJ, Finarelli JA, Zehr S, Hsu J, Nedbal MA. 2005. Molecular phylogeny of the carnivora (mammalia): assessing the impact of increased sampling on resolving enigmatic relationships. *Syst Biol.* 54:317-37.
- Govett, P.D., Harms, C.A., Johnson, A.J., Latimer, K.S., Wellehan, J.F., Fatzinger, M.H., Christian, L.S., Kelly, T.R., Lewbart, G.A., 2005. Lymphoid follicular cloacal inflammation associated with a novel herpesvirus in juvenile alligators (*Alligator mississippiensis*). *J. Vet. Diagn. Invest.* 17, 474-479.
- Hanson, L.A., Rudis, M.R., Vasquez-Lee, M., Montgomery, R.D. 2006. A broadly applicable method to characterise large DNA viruses and adenoviruses based on the DNA polymerase gene. *Virology* 3:28.
- Havird JC1, Miyamoto MM. 2010. The importance of taxon sampling in genomic studies: an example from the cyclooxygenases of teleost fishes. *Mol Phylogenet Evol.* 56:451-5
- Jacobson, E.R. 2007. Viruses and Viral Diseases of Reptiles. In: Jacobson, E.R. (Ed.), *Infectious Diseases and Pathology of Reptiles*. Taylor & Francis Group, LLC, Boca Raton, FLA, pp. 395-460.
- Jacobson E.R., Gaskin J.M., Roelke M., Breiner E.C., Allen J. 1986. Conjunctivitis, tracheitis, and pneumonia associated with herpesvirus infection in green sea turtles. *J. Am. Vet. Med. Assoc.* 189, 1020-1023.

- Jungwirth, N., Bodewes, R., Osterhaus, A.D.M.E., Baumgärtner, W., Wohlsein, P. 2014. First report of a new alphaherpesvirus in a freshwater turtle (*Pseudemys concinna concinna*) kept in Germany. *Vet. Microbiol.* 170, 403-407.
- Katoh, K., Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform.* 9, 286-298.
- Marschang R.E., Gleiser C.B., Papp T., Pfitzner A.J., Böhm R., Roth B.N. 2006. Comparison of 11 herpesvirus isolates from tortoises using partial sequences from three conserved genes. *Vet. Microbiol.* 117, 258-266.
- Marschang, R.E., 2011. Viruses infecting reptiles. *Viruses* 3, 2087-2126.
- McCowan, C., Shepherdley, C., Slocombe, R.F., 2004. Herpesvirus-like particles in the skin of a saltwater crocodile (*Crocodylus porosus*). *Aust. Vet. J.* 82, 375-377.
- McGeoch, D.J., Gatherer, D. 2005. Integrating Reptilian Herpesviruses into the Family *Herpesviridae*. *J. Virol.* 79, 725-731.
- Miller M.A., Pfeiffer W., Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), 14 November, New Orleans, Louisiana, pp. 1-8.
- Origi, F.C. 2006. Herpesviruses in Tortoises. In: Mader D.R. (Ed). *Reptile Medicine and Surgery*, 2nd ed., Saunders Elsevier, St. Louis, MO. pp. 814-821
- Pellet P., Roizman B. 2007. The family Herpesviridae: a brief introduction. In: *Fields' virology*, 5th Ed. Knipe, D., Howley, P.M. (Eds.). Lippincott Williams & Wilkins, Philadelphia, PA. pp. 2479-2499.
- Pellet, P.E., Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., Lacoste, V., Minson, A.C., Nicholas, J., Roizman, B., Studdert, M.J., Wang, F. 2012. Herpesvirales, In: King,

- A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.) Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Academic Press, London, UK, 99-123.
- Ronquist F., Huelsenbeck J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572-1574.
- Stamatakis A, Hoover P, Rougemont J: 2008, A rapid bootstrap algorithm for the RAxML Web Servers. *Syst Biol* 57:758-771.
- Susko, E. 2009. Bootstrap Support Is Not First-Order Correct. *Systematic Biology* 58:211-223.
- VanDevanter, D.R., Warrener, P., Bennett, L., Schultz, E.R., Coulter, S., Garber, R.L., Rose, T.M., 1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. *J Clin Microbiol* 34, 1666-1671.
- Whelan S., Goldman N. 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* 18, 691-699.
- Wellehan J.F.X., Nichols D.K., Li L., Kapu V. 2004. Three novel herpesviruses associated with stomatitis in sudan plated lizards (*Gerrhosaurus major*) and a black-lined plated lizard (*Gerrhosaurus nigrolineatus*). *J. Zoo Wildl. Med.* 35, 50-54.

Figure Captions

(a)



(b)

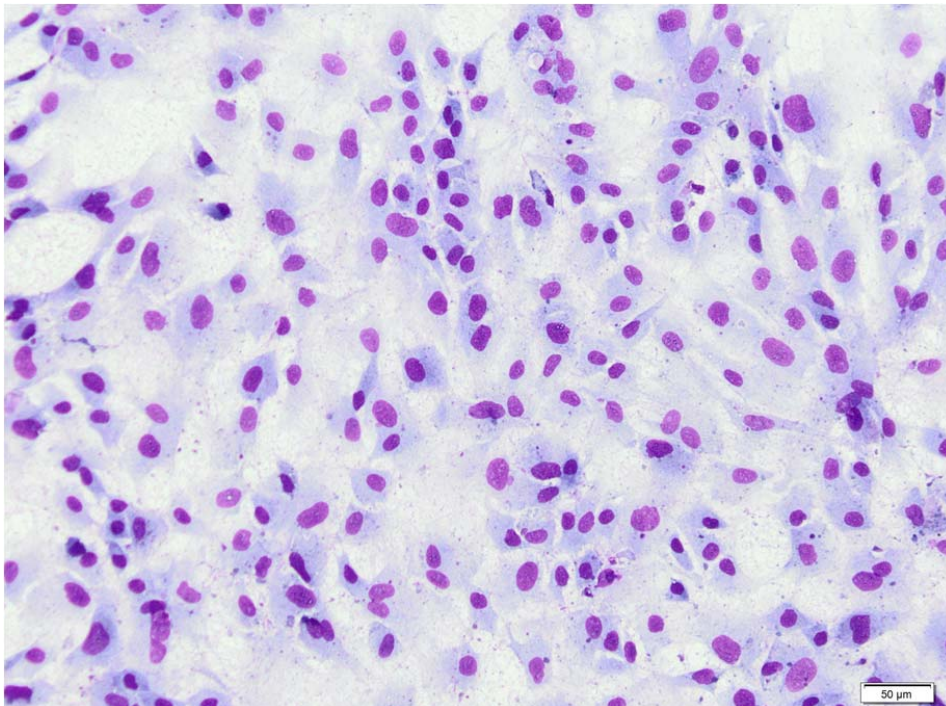


Figure 1. Giemsa-stained primary cell lines derived from saltwater crocodile hatchlings less than 24 h old. Cells infected with Crocodyline herpesvirus 2 (A) show loss of confluence of the cell monolayer, cell rounding and syncytial cell formation. Uninfected cells (B).

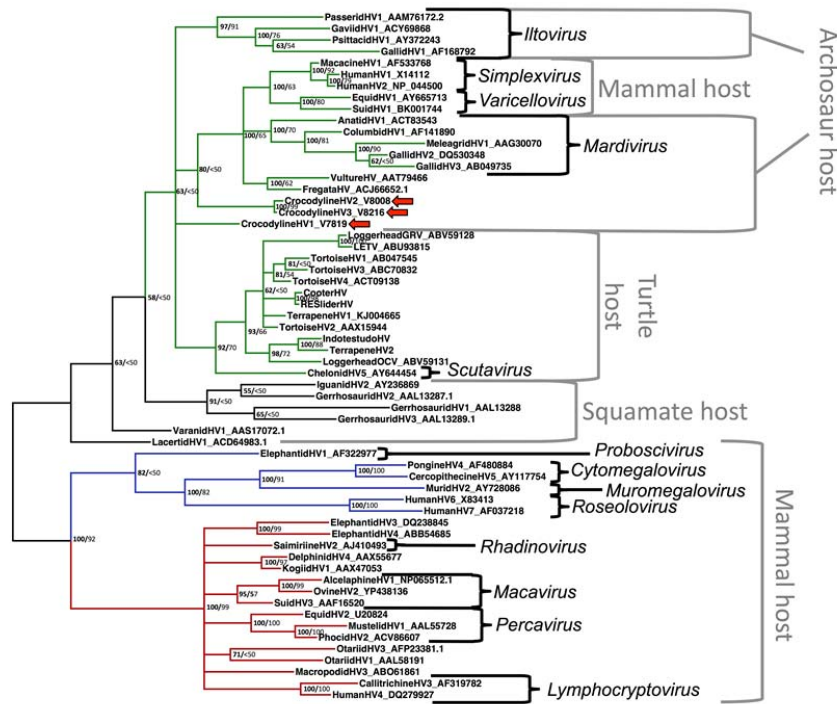


Figure 2. Bayesian phylogenetic tree of predicted amino acid sequences of herpesviral DNA-dependent DNA polymerase sequences based on MAFFT alignment. Bayesian posterior probabilities of clusters are given at nodes in bold on the left, and maximum likelihood bootstrap values are on the right. Lacertid herpesvirus 1 was used as an outgroup. The crocodyline herpesviruses are indicated with arrows. The subfamily *Alphaherpesvirinae* is marked in green, *Betaherpesvirinae* is in blue, and *Gammaherpesvirinae* is in red. Brackets demarcate genera. Accession numbers of sequences retrieved from GenBank are given after the name.

Tables

Table 1. Summary of herpesvirus PCR and sequencing results in Australian farmed saltwater crocodiles (*Crocodylus porosus*) and captive freshwater crocodiles (*C. johnstoni*).

Disease Syndrome	Origin of Animal	Sample: I = isolate T = tissue ¹	Pan-Herpesvirus PCRs		Croc herpesvirus PCR	Herpesvirus PCR product sequence
			VanDevanter PCR	Hanson PCR		
<i>Saltwater Crocodile Cases</i>						
Conjunctivitis-Pharyngitis (CP)	Northern Territory (NT), Farm 1	I: Conjunctiva	Positive	Positive	Positive	CrHV2
CP	NT, Farm 2	I: Conjunctiva	Positive	Positive	Positive	CrHV1
CP	NT, Farm 1	I: Conjunctiva	Positive	Positive	Positive	CrHV1
		T: Eye, pharynx, lung, thymus	Negative	Negative	Positive	CrHV1
CP with concurrent skin ulcers	NT, Farm 1	I: Skin	Positive	Positive	Positive	CrHV2
Systemic lymphoid proliferation and encephalitis (SLPE)	NT, Farm 2	I: Liver	Positive	Positive	Positive	CrHV2
		T: Lung, heart, liver, spleen, pancreas, intestine	Negative	Negative	Positive	CrHV2
SLPE	NT, Farm 2	I: Kidney	Positive	Positive	Positive	CrHV2
		T: Lung, heart, thymus, brain, oesophagus	Negative	Negative	Positive	CrHV2
SLPE	NT, Farm 2	I: Kidney	Negative	Positive	Positive	CrHV2
Lymphnodular skin (LNS)	NT, Farm 2	I: Skin	Positive	Positive	Positive	CrHV2

Freshwater Crocodile Cases

Freshwater crocodile systemic lymphoid proliferation (FSLP)	NT, Farm 1	I: Liver	Negative	Positive	Positive	CrHV3
FSLP	NT, Farm 1	I: Lung	Negative	Negative	Negative	n.a.
		T: Liver, kidney, spleen, fat body	Negative	Negative	Positive	CrHV3

Isolate Positives / Total Isolates Tested

7/10 9/10 9/10

FFPE Positives / Total FFPE Tested

0/4 0/4 4/4

Controls

Saltwater crocodile cell culture	NT	Spent media of non-inoculated cells	Negative	Negative	Negative	n.a.
Freshwater crocodile. Chronic bacterial dermatitis, nematodiasis and septicaemia	Western Australia (WA)	T: liver, spleen, lung	Negative	Negative	Negative	n.a.
Saltwater crocodile. History of severe hypothermia. No gross or histological lesions noted.	WA	T: liver, lung, kidney	Negative	Negative	Negative	n.a.
Equine herpesvirus 1		Laboratory isolate	Positive	Positive	Negative	n.a.
Feline herpesvirus 1		Vaccine	Positive	Negative ²	Negative	n.a.
Gallid herpesvirus 2 (Marek's disease virus)		Vaccine	Positive	Positive	Negative	n.a.

¹ All tissues were formalin-fixed paraffin-embedded (FFPE).

² This result was obtained twice.

n.a. = not applicable.

Table 2. Summary of PCRs used in this study.

PCR Type	Primer Sequences, 5' → 3'	PCR Product Size (bp)		Reference
		Crocodyline herpesvirus 1	Crocodyline herpesvirus 2 & 3	
Nested*	DFA: GAYTTYGCNAGYYTNTAYCC ILK: TCCTGGACAAGCAGCARNYSGCNMTNAA KG1: GTCTTGCTCACCAGNTCNACNCCYTT TGV: TGTAACTCGGTGTAYGGNTTYACNGGNGT IYG: CACAGAGTCCGTRTCNCCRTADAT	222	234	VanDevanter et al., 1996
Single round	DNA Virus S: CGGAATTCTAGAYTTYGCNWSNYTNTAYCC DNA Virus AS: CCCGAATTCAGATCTCNGTRTCNCCRTA	530	542	Hanson et al., 2006
Single round duplex	Croc Herpes 1 FW: CTCCACCTGCGGGAAGCTC Croc Herpes 1 RV: GCCACCGTCACCACCATC Croc Herpes 2/3 FW: CACTTGCTGCTTCGGGAAAC Croc Herpes 2/3 RV: GGCCACCGTGACCACTATAG	108	113	Present Study

* First round = primers DFA, ILK and KG1. Second Round = primers TGV and IYG.

Table 3. Sequence identities of a 472-484 nucleotide and 153-161 amino acid segment of DNA-dependent DNA polymerase from three crocodile herpesviruses (GenBank accession numbers KP313603, KP313604 and KP313605).

Virus	Nucleotide		Virus	Amino Acid	
	CrHV1	CrHV2		CrHV1	CrHV2
CrHV2	58.3%	-	CrHV2	65.6%	-
CrHV3	58.3%	94.6%	CrHV3	66.9%	96.3%