Veterinary Microbiology 181 (2015) 183–189

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

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,*], Catherine M. Shilton[b], James F.X. Wellehan Jr[c], Steven Davis[b], Sally R. Isberg[d,e], David Phalen[d], Lorna Melville[b]

[a] Murdoch University, School of Veterinary and Life Sciences, Murdoch, Western Australia 6150, Australia
[b] Berrimah Veterinary Laboratories, Northern Territory Government, GPO Box 3000, Darwin, Northern Territory 0801, Australia
[c] Department of Small Animal Clinical Sciences, University of Florida College of Veterinary Medicine, Gainesville, Florida 32610, USA
[d] The University of Sydney, Sydney, New South Wales 2006, Australia
[e] Centre for Crocodile Research, Noonamah, Northern Territory 0837, Australia

ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 4 September 2015
Accepted 16 September 2015

Keywords:
Reptile
Alphaherpesvirus
Virus isolation
Phylogeny
PCR

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 (*Crocodylus johnstoni*), 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.

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; 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; Origgi, 2006; Wellehan et al., 2004).

* Corresponding author.
E-mail addresses: t.hyndman@murdoch.edu.au (T.H. Hyndman), cathy.shilton@nt.gov.au (C.M. Shilton), wellehanj@ufl.edu (J.F.X. Wellehan), steven.davis@nt.gov.au (S. Davis), sally@crocresearch.com.au (S.R. Isberg), david.phalen@sydney.edu.au (D. Phalen), lorna.melville@nt.gov.au (L. Melville).

http://dx.doi.org/10.1016/j.vetmic.2015.09.013
0378-1135/

Herpesviruses in crocodilians 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 (*C. 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 h 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 min 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 cm^2 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 cm^2 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 (Fig. 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 visualized using 0.005% SYBR safe (v/v; Invitrogen, Mulgrave, Victoria) and a transilluminator (DR88M Dark Reader non-UV Transilluminator; Clare Chemical Research