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Herpesvirus Infection in Lumholtz's Tree-kangaroo (*Dendrolagus lumholtzi*)

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ABSTRACT: Herpesvirus infections associated with a range of clinical findings are widespread in free-ranging and captive Australian marsupials. We report on herpesviruses identified by virus neutralization and PCR in free-ranging and captive Lumholtz's tree-kangaroos (*Dendrolagus lumholtzi*). Herpesvirus has not been confirmed previously by DNA testing in tree kangaroos. Virus neutralization testing for alphaherpesviruses MaHV1 and MaHV2 was positive on 4/10 captive and 0/35 free-ranging tree-kangaroo samples tested. A novel gammaherpesvirus was found on PCR in 17/20 apparently healthy individuals (11/12 free-ranging, 5/6 wild-caught, captive, and 1/2 captive-bred). One captive-bred animal that died following an acute illness was positive on PCR only for MaHV4, an alphaherpesvirus previously identified from an eastern grey kangaroo (*Macropus giganteus*). The detection of MaHV4, associated with morbidity and mortality in captive tree-kangaroos, raises biosecurity concerns about introducing a non-endemic alphaherpesvirus into naive wild populations through release of captive animals. We propose that: 1) further work on herpesviruses in marsupials be carried out to determine whether herpesviruses from captive individuals represent a potential threat to wild populations, particularly for endangered species in which there are captive breeding and cross-fostering programs; and 2) that captive tree kangaroos be kept in such a way that prevents cross-species transmission of herpesviruses, in particular eliminating close direct or indirect contact with other species of macropods.

Key words: Biosecurity, captive management, *Dendrolagus*, herpesvirus.

Herpesvirus infection is widespread in a range of Australian marsupials with four viruses (the alphaherpesviruses, MaHV1, MaHV2, MaHV4, and the gammaherpesvirus, MaHV3) associated with clinical disease and mortalities, primarily in captive animals (Stalder et al. 2015). Lumholtz's tree-kangaroo (*Dendrolagus lumholtzi*), one of two species of tree-kangaroos found in Australia, is the only Australian tree-kangaroo species kept in captivity. A 2014–19 survey into the free-ranging population collected data on demographics, general health, physical parameters, and reproductive status from 192 individuals (40 live and 152 dead) from various sites on the Atherton Tablelands, Queensland, Australia. The investigation reported here, utilizing a subset of 60 samples from 59 individuals (44 free-ranging, 13 wild-caught, captive, and 2 captive-bred) was initiated after finding intranuclear inclusion bodies (INIB) consistent with herpesvirus in the liver of a captive-bred animal (FC5) that died following an acute illness. Histopathology, virus neutralization serology (VN) and PCR of swabs and postmortem tissue samples were used to assess the herpesvirus status in Lumholtz's tree-kangaroo. Because this work was started after the population health survey was already underway, the datasets for VN versus PCR vary, based on what samples were available (Supplementary Material Table S1). Virus neutralization was used to assay serum from 45 individuals in total, 35 for which no material was available for PCR. Ten were tested by VN and PCR and are included in the PCR results reported in the upcoming text and in Table 1.

Virus neutralization assays against alphaherpesviruses MaHV1 and MaHV2 were performed as described previously (Vaz et al. 2013) at a 1:10 dilution with cultures examined after 24–48 h incubation for the presence of cytopathic effects. None of the 35 free-ranging but 44% (4/9) captive animals were seropositive. All four VN-positive captive animals had a history of having been paired up and housed together for breeding.

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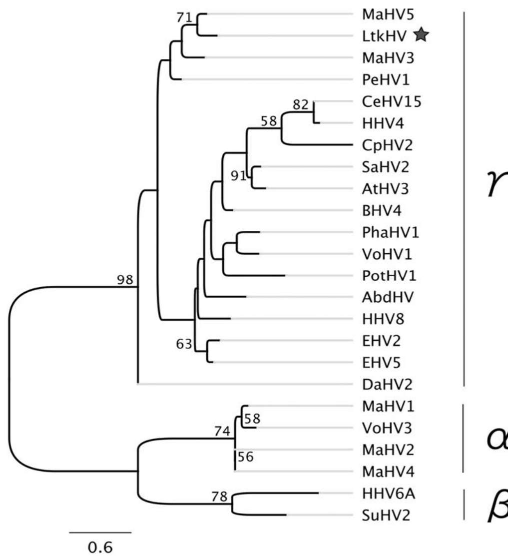

FIGURE 1. Comparison of the novel Lumholtz's tree-kangaroo (*Dendrolagus lumholtzi*) gammaherpesvirus (LtkHV; marked by a star) with representative members of the three *Herpesviridae* subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Maximum-likelihood phylogenetic tree was generated using predicted amino acid sequences using a Jones-Taylor-Thorton model of amino acid replacement with four substitution rates per category. Bootstrapping values (n=100) higher than 55 are shown for each branch. The abbreviations and GenBank accession details or reference for sequences are: macropodid gammaherpesvirus 3 (MaHV3; GenBank no. EF467663.1), cercopithecine gammaherpesvirus 15 (CeHV15; GenBank ALF08184.1), human gammaherpesvirus 4 (HHV4; GenBank YP_401712.1), caprine gammaherpesvirus 2 (CpHV2; GenBank ADY17130.1), saimirine gammaherpesvirus 2 (SaHV2; GenBank NP_040211.1), ateline gammaherpesvirus 3 (AtHV3; GenBank NP_047983.1), bovine gammaherpesvirus 4 (BHV4; GenBank NP_076501.1), phascolarctid gammaherpesvirus 1 (PhaHV1; GenBank JN585829), vombatid gammaherpesvirus 1 (VoHV1; GenBank AZB49114.1), Atlantic bottlenose dolphin gammaherpesvius (AbdHV; GenBank ABC33906.1), human gammaherpesvirus 8 (HHV8; GenBank YP_001129355.1), equine gammaherpesvirus 2 (EHV2; GenBank NP_042605.1), equine gammaherpesvirus 5 (EHV5; GenBank YP_009118399.1), macropodid alphaherpesvirus 1 (MaHV1; Genbank YP_009227263.1), human betaherpesvirus 6 (HHV6A; GenBank NP_042931.1), and suid betaherpesvirus 2 (SuHV2 GenBank YP_008492977.1). Sequences for macropodid gammaherpesvirus 5 (MaHV5), peramelid gammaherpesvirus 1 (PeHV1), vombatid alphaherpesvirus 3 (VoHV3), and dasyurid gammaherpesviurs 2 (DaHV2) were extracted from Stalder et al. (2015) and potoroid gammaherpesvirus 1 (PotHV1) from ← Portas et al. (2014) and are shown in Supplementary Material Figure S1. Sequences for LtkHV, macropodid alphaherpesvirus 2 (MaHV2), and macropodid herpesvirus 4 (MaHV4) are available in Supplementary Text ST1.

We used PCR to survey for herpesvirus DNA in 20 individuals (Table 1). Swabs from tissues (conjunctiva, oral cavity, nasal passages, and/or cloaca) were collected and stored at –20 C. The DNA from these samples was extracted using VX Universal Liquid Sample DNA Extraction kit (Qiagen, Hilden, Germany) and a Corbett X tractor Gene Robot (Corbett Robotics/Qiagen, Hilden, Germany). We extracted DNA from formalin-fixed paraffin-embedded (FFPE) tissue by initial deparaffinization in xylene, followed by ethanol and phosphate-buffered saline washes, and finally through the use of a Promega Genomic DNA purification kit (Promega, Madison, Wisconsin, USA). Sample extracts were tested for herpesvirus DNA using a pan-herpesvirus nested PCR that generates amplicons of approximately 200 base pairs, targeting a conserved region of the DNA polymerase gene (VanDevanter et al. 1996; Chmielewicz et al. 2003). Amplified PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Foster City, California, USA) and sequences were compared with published sequences in the GenBank database using the BLAST-X online algorithm (Benson et al. 2013). Clustal W2 (Larkin et al. 2007) was used to compare the nucleotide and predicted amino acid sequences of the amplified products to other known marsupial herpesviruses, including those that were not available on the GenBank database (Stalder et al. 2015). Comparison of the unknown gammaherpesvirus to representative members of the *Herpesviridae* family was performed using the predicted amino acid sequence alignment (57 amino acid length; Fig. 1). A maximum likelihood phylogenetic tree was generated using a Jones-Taylor-Thornton model of amino acid replacement with four substitution rate categories (Jones et al. 1992). Other

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TABLE 1. Results for virus neutralization (VN) for antibodies for alphaherpesviruses, macropodid herpesviruses 1 and 2 (MaHV1, 2) from nine individuals (10 samples; one individual tested twice), and PCR results from n=20 (n=12 free-ranging; n=6 wild-caught, captive; n=2 captive bred) Lumholtz's tree-kangaroo (*Dendrolagus lumholtzi*). Results from n=35 individuals (n=21 wild, live; n=11 wild, postmortem roadkill; n=3 captive) for which only VN testing was performed, and which were all negative on VN testing are not shown in this table. An additional five formalin-fixed paraffin embedded (FFPE) samples (all negative on PCR for herpesvirus) from individuals for which no background clinical information was available are also not shown in this table.

			Swab sample site[a]					
Status[b]	Identification	PCR result[c]	Ocular conjunctiva	Oral cavity	Nasal tissue	Cloaca	Virus neutralization	Comments
W[a]	WM12	LtkHV	NA	Positive	Positive	Negative	Negative	
W	FC1	LtkHV	Positive	Positive	NA	Negative	Negative	
W	MC9	Negative	Negative	Negative	Negative	Negative	Negative	Under 2-yr-old
dW[d]	DA431	LtkHV	Positive	Positive	NA	Negative	NA	
dW	DA442	LtkHV	NA	Positive	Negative	NA	NA	
dW	DA448	LtkHV	NA	Positive	Positive	NA	NA	
dW	DA444	LtkHV	Positive	Positive	Positive	Negative	NA	
dW	DA413	LtkHV	Positive	NA	Positive	NA	NA	
dW	DA449	LtkHV	Positive	Positive	NA	NA	NA	
dW	DA441	LtkHV	Positive	Positive	Positive	Negative	NA	
dW	DA445	LtkHV	Positive	Positive	Positive	Positive	NA	
dW	DA447	LtkHV	Positive	NA	Positive	Positive	NA	
C[e]	FC3	LtkHV	NA	Positive	Positive	Negative	Positive	Mild respiratory signs July 2016; dam of FC5
C	MC12	LtkHV	Negative	Positive	Negative	Negative	Negative	
C	MC13	LtkHV	Negative	Positive	Negative	Negative	Negative	
C	MC5	LtkHV	Positive	Positive	Positive	Negative	Positive	There are two samples from this individual: virus neutralization negative in December 2014; housed with FC4 in January 2015; virus neutralization positive when retested in November 2017; intranuclear inclusion bodies consistent with herpesvirus in liver on histopathology.
C	MC14	LtkHV	Negative	Positive	Positive	Negative	Negative	
C	FC4	Negative[d]	NA	NA	NA	NA	Positive	Died August 2015; PCR done on FFPE tissue only
Cb[g]	MC1	LtkHV	NA	Positive	Positive	Negative	Positive	VN-positive December 2014; PCR done May 2017; sire of FC5

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Table 1. Continued.

Status[b]	Identification	PCR result[c]	Swab sample site[a] Ocular conjunctiva	Oral cavity	Nasal tissue	Cloaca	Virus neutralization	Comments
C	FC5	MaHV4	NA	NA	MaHV4	NA	NA	Under 3-yr-old; died June 2016; offspring of FC3; herpesvirus inclusion bodies seen in liver on histopathology

[a] NA = not available.
[b] W = free-ranging; dW = roadkill free-ranging; C = captive, wild-caught; Cb = captive bred.
[c] LtkHV = unknown gammaherpesvirus; MaHV4 = macropodid alphaherpesvirus 4.
[d] PCR on FFPE tissue only.

models were tested but no significant topological differences were observed. Bootstrap support values (n=100) higher than 55 are shown for each branch (Felsenstein 1985). All FFPE tissues from six wild-caught, captive individuals were negative on PCR. Historical background information on the condition of the samples and circumstances surrounding the deaths was unavailable for five individuals. so results from only one individual (FC4) for which clinical history was known were included in the dataset in Table 1. Herpesvirus positive control Lumholtz's tree-kangaroo FFPE tissue samples were not available to validate PCR results so it is not possible to ascertain whether the negative PCR results on FFPE tissues were a result of technical problems or due to sufficient viral DNA not being present.

An unknown gammaherpesvirus, which we propose to identify as LtkHV, was detected by PCR in swab samples from 85% (17/20) of the animals tested (Table 1). The oral cavity was the site most consistently positive on PCR (Table 1). The DNA sequence (Supplementary Text ST1) of the PCR products most closely matched the gammaherpesvirus MaHV5 from the swamp wallaby (*Wallabia bicolor*) with 77% amino acid identity. No evidence of association with clinical disease or mortality was seen in LtkHV-positive animals, with the exception of three individuals (MC1, MC5, and FC3) that, based on VN results, were coinfected with an alphaherpesvirus (Table 1). Gammaherpesviruses do not show serologic cross-reactivity with VN testing for alphaherpesviruses MaHV1, 2 (Stalder et al. 2015).

Two captive individuals were found with INIB consistent with herpesvirus on histopathology of postmortem liver samples. One (FC5) was PCR positive for MaHV4 on postmortem nasal tissue swabs (Table 1). This individual died after an acute illness, initially presenting with acute respiratory signs (dyspnea, tachypnea, and moist rales) and oculonasal discharge. The second individual, an adult wild-caught male (MC5), showed clinical signs of illness (tachypnea, dyspnea, lethargy) 2 wk after being housed with FC4 but appeared to recover with antibiotic therapy. He continued with sporadic episodes of illness showing clinical signs including oculonasal discharge, inappetence, lethargy, blistering of food pads, and self-mutilation of his tail, eventually culminating in his death in 2019. This individual had been seronegative for MaHV1 and MaHV2 when tested in 2014 but when retested 225 d later, after being housed for breeding with female FC4 (who was VN seropositive for MaHV1 and MsHV2) had seroconverted (Table 1). Serologic cross-reactivity between macropodid alphaherpesviruses has been demonstrated so further

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testing (for which samples are unavailable) would be required to ascertain whether the VN positive results were due to MaHV1, MaHV2, or MaHV4 infection (Vaz et al. 2013; Stalder et al. 2015). Histopathology revealed INIB consistent with herpesvirus in liver cells. Previously, MaHV4 has been identified only in free-ranging eastern grey kangaroos (*Macropus giganteus*; Vaz et al. 2013). The detection of MaHV4 associated with morbidity and acute mortality in a juvenile captive tree-kangaroo (FC5) raises concerns that, although adult tree kangaroos might be capable of surviving infection with alphaherpesvirus, it could be highly pathogenic in juveniles.

Comingling with other species in captivity could result in infection with nonendemic herpesviruses that have the potential to cause disease and mortality, especially when animals are under stress from captivity or following reintroduction to the wild (Schrenzel et al. 2003). In our study, evidence of alphaherpesvirus infection was only found in captive tree-kangaroos and might have been the result of exposure while being cohoused (a common practice in the wildlife care network) with other species which might be the natural hosts for the alphaherpesviruses. The possibility that MaHV4 could be introduced into a naïve, free-ranging population of tree-kangaroos through the release of animals that have been in captivity must be considered in management decisions for the species. We propose that captive tree-kangaroos be prevented from direct or indirect contact with other species of macropods. Prior to release of a tree-kangaroo back into the wild, serologic testing for alphaherpesvirus is warranted, particularly if the individual has not been kept in isolation from other macropod species. Due to the ability of herpesvirus to remain latent and shed only intermittently, PCR testing only reveals active infection/shedding of alphaherpesvirus, not latent infection that can recrudesce and cause clinical signs, so VN testing might be the only reliable method for prerelease screening for exposure to alphaherpesvirus in tree-kangaroos. We recommend surveying to establish whether alphaherpesvirus (especially MaHV4) occurs in the red-legged pademelon (*Thylogale stigmatica*), and other species cohoused with captive and rescued tree-kangaroos.

Our study highlights the potential biosecurity risk posed by release of rescued wildlife, particularly for endangered species recovery and release programs. Given limited resources, careful consideration should be given to practical and effective means of maintaining good biosecurity and monitoring the release of rescued wildlife to minimize the potential for unintended negative impacts on wild populations.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2019-07-184.

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