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SHORT COMMUNICATION

Molecular detection of two new putative species of gammaherpesvirus in petaurid possums

JK Douch, JM Devlin, P Whiteley, CA Hartley* and PK Vaz

A molecular survey of herpesviruses in Australian native mammals was conducted, spanning 260 individuals from 27 species. Among the herpesviruses detected, a putative new gammaherpesvirus species was detected in the yellow-bellied glider (*Petaurus australis*), and another in the critically endangered Leadbeater's possum (*Gymnobelideus leadbeateri*). In addition, the known host range of the putative species macropodid gammaherpesvirus 3 (MaHV-3) is herein extended to the western grey kangaroo (*Macropus fuliginosus*). These findings expand our understanding of herpesviruses in Australian mammals and may inform biosecurity protocols for captive and translocated populations.

Keywords *Gammaherpesvirinae*; *Herpesviridae*; marsupial; wildlife

Abbreviations DPOL, DNA polymerase; LPHV, Leadbeater's possum gammaherpesvirus; UHPCR, universal herpesvirus polymerase chain reaction; YBGHV, yellow-bellied glider gammaherpesvirus

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Herpesviridae is a family of more than 100 member species of double-stranded DNA viruses that infect amniotes. Herpesvirus infections are characterised by an acute primary infection followed by the establishment of a latent infection. Reactivation of latent infections can result in shedding of virus either with or without clinical signs, so that intermittent periods of virus shedding and transmission can occur throughout the host's lifetime. The clinical consequences of herpesvirus infection can range from no signs of disease to rapid mortality. Herpesvirus infections in humans and domestic animals have been demonstrated to cause severe morbidity and, in the case of the latter, economic losses. There are also numerous unique herpesvirus species or putative species hosted by a wide range of native Australian animals, including marsupials.[1] For example, infection with *Macropodid alphaherpesvirus 1* is associated with conjunctivitis, rhinitis, cloacal lesions and pneumonia in parma wallabies (*Macropus parma*).[2] Wildlife translocation and co-housing of captive animals leads to stress and increases the opportunity for reactivation. Shedding and transmission can occur from natural hosts with or without clinical signs, to infect potentially vulnerable new host species, as has occurred previously among Australian macropods.[3] Therefore, an accurate and comprehensive understanding of the herpesviruses that infect these hosts will be a valuable information to help inform biosecurity protocols.

This report describes the results of a survey for herpesviruses in Australian native mammals from free ranging and captive populations. Many possums (n = 119) were included, as well as eastern grey kangaroos (*Macropus giganteus*; n = 52), koalas (*Phascolarctos cinereus*; n = 20), and southern brown bandicoots (*Isoodon obesulus*; n = 20). Smaller numbers of other native mammal species were included. In total the survey spanned 258 individuals from 25 species (Table 1).

Samples were opportunistically used in this survey, collected for other clinical investigations by veterinary professionals or as components of other research and surveillance projects (including DELWP permit #10009447).[4] Samples included cloacal, nasal, ocular and oropharyngeal swabs that were stored dry, in phosphate-buffered saline solution, or in viral transport medium.[1] Blood, spleen and other tissues were also received that had been excised postmortem. All samples were preserved at −20 or −80°C until analysis, depending on the availability of ultra-low temperature freezers at the location of collection.

For swabs, DNA extraction was performed with the QIAamp Viral RNA Mini Kit (QIAGEN) or the MagMAX CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific). For tissues, the Wizard SV Genomic DNA Purification System (Promega) was used instead. Extracted samples were screened for the presence of herpesvirus DNA via a nested pan-herpesvirus polymerase chain reaction (UHPCR) assay, which targets a conserved region of the DNA polymerase (DPOL) catalytic subunit, U_L30.[5] Successful amplification of second-round products of the expected size (~200 bp) was determined by electrophoresis on 2% w/v agarose gel stained with SYBR Safe (Thermo Fisher Scientific). Amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN) and a sequencing reaction was performed using BigDye Terminator v3.1 (Thermo Fisher Scientific). Sequencing reactions were submitted to the Australian Genome Research Facility, Melbourne, for Sanger sequencing. Manufacturers' protocols were followed in each case.

The DNA sequences generated in this study were queried using BLASTp in GenBank (National Center for Biotechnology Information)[6] to find the closest available sequences in the database. In addition, published DPOL sequences not available in GenBank (due to their <200 bp size) were also included in the analysis.[1, 3, 7–9]

*Corresponding author.
Asia Pacific Centre for Animal Health, Department of Veterinary Biosciences, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Melbourne, Victoria, Australia; carolah@unimelb.edu.au

Table 1. Universal herpesvirus polymerase chain reaction (UHPCR) results, as determined by gel electrophoresis and nucleotide sequencing

Species sampled	Total individual animals tested	Positive individuals	Herpesvirus detected	Sample type	Clinical signs of disease (n)[a]
Agile antechinus (*Antechinus agilis*)	1	0	—	Swab	—
Brush-tailed phascogale (*Phascogale tapoatafa*)	2	0	—	Swab	—
Brush-tailed rock wallaby (*Petrogale penicillata*)	5	0	—	Spleen	—
Common brushtail possum (*Trichosurus volpecula*)	48	0	—	Swab and spleen	2
Common ringtail possum (*Pseudochirus peregrinus*)	67	0	—	Swab and spleen	2
Common wombat (*Vombatus ursinus*)	2	1	VoHV-2	Swab	—
Eastern barred bandicoot (*Perameles gunii*)	2	0	—	Swab and buffy coat	—
Eastern grey kangaroo (*Macropus giganteus*)	52	13	MaHV-3 (n = 12), MaHV-4 (n = 1)	Swab	8[b]
Eastern quoll (*Dasyurus viverrinus*)	5	5	DaHV-4[c]	Swab	—
Greater glider (*Petauroides volans*)	1	0	—	Swab and lung	—
Koala (*Phascolarctos cinereus*)	20	6	PhaHV-1 (n = 3), PhaHV-2 (n = 3)	Swab	4[d]
Leadbeater's possum (*Gymnobelideus leadbeateri*)	4	2	LPHV	Multiple tissues	—
Long-nosed potoroo (*Potorous tridactylus*)	5	0	—	Buffy coat	—
Northern nail-tail wallaby (*Onychogalea unguifera*)	1	0	—	Swab	—
Parma wallaby (*Macropus parma*)	2	0	—	Swab	—
Red-necked wallaby (*Macropus rufogriseus*)	1	0	—	Swab	—
Platypus (*Ornithorhynchus anatinus*)	8	0	—	Swab and multiple tissues	1
Short-beaked echidna (*Tachyglossus aculeatus*)	1	0	—	Swab	—
Southern brown bandicoot (*Isoodon obesulus*)	20	0	—	Swab	—
Southern hairy-nosed wombat (*Lasiorhinus latifrons*)	1	0	—	Liver	—
Sugar glider (*Petaurus breviceps*)	3	0	—	Swab	—
Swamp wallaby (*Wallabia bicolor*)	4	0	—	Swab and multiple tissues	—
Western grey kangaroo (*Macropus fuliginosis*)	1	1	MaHV-3	Swab	1[e]
Yellow-bellied glider (*Petaurus australis*)	1	1	YBGHV	Buffy coat	—
Yellow-footed antechinus (*Antechinus flavipes*)	1	0	—	Swab and multiple tissues	—

[a] Where known.
[b] 6 of these 8 *M. giganteus* individuals positive for MaHV-3.
[c] The two UHPCR-positive samples were identified as DaHV-4 on the basis of High-Resolution Melt only;
[d] 1 of these 4 *P. cinereus* individuals positive for PhaHV-2.
[e] *M. fuliginosis* individual positive for MaHV-3.
DaHV-4, dasyurid gammaherpesvirus-4; LPHV, Leadbeater's possum herpesvirus; MaHV-3, macropodid gammaherpesvirus-3; MaHV-4, macropodid alphaherpesvirus-4; PhaHV-1, *Phascolarctid alphaherpesvirus-1*; PhaHV-2, phascolarctid gammaherpesvirus-2; VoHV-2, vombatid gammaherpesvirus-2; YBGHV, yellow-bellied glider herpesvirus.

Nucleotide sequences generated in this study were translated and aligned with trimmed amino acid sequences using Multiple Sequence Comparison by Log-Expectation[10] on default settings in Geneious Prime 2020.2.3,[11] and a maximum-likelihood phylogeny was produced using the Geneious plugin PhyML, on default settings with 100 bootstrap iterations.[12]

The UHPCR survey detected multiple previously known herpesvirus species (Table 1) in five animal species. Among these, the putative species macropodid gammaherpesvirus 3 (MaHV-3) was detected for the first time in a western grey kangaroo (*Macropus fuliginosus*); a captive individual of the Kangaroo Island subspecies (*M. fuliginosus fuliginosus*; Table 1) with intermittent chronic ocular discharge, sampled in 2021. Prior to this, the eastern grey kangaroo was the only known host of MaHV-3.[13] The two species are partially sympatric, but it is unknown whether wild western grey kangaroos host this virus, or whether a case of spill-over within captivity has been encountered. All swabs collected from the western grey kangaroo (from cloaca, nose, oropharynx and a combination of the three) tested positive.

The UHPCR survey also detected two potentially novel herpesviruses. The DPOL sequences identified in both the yellow-bellied glider sample

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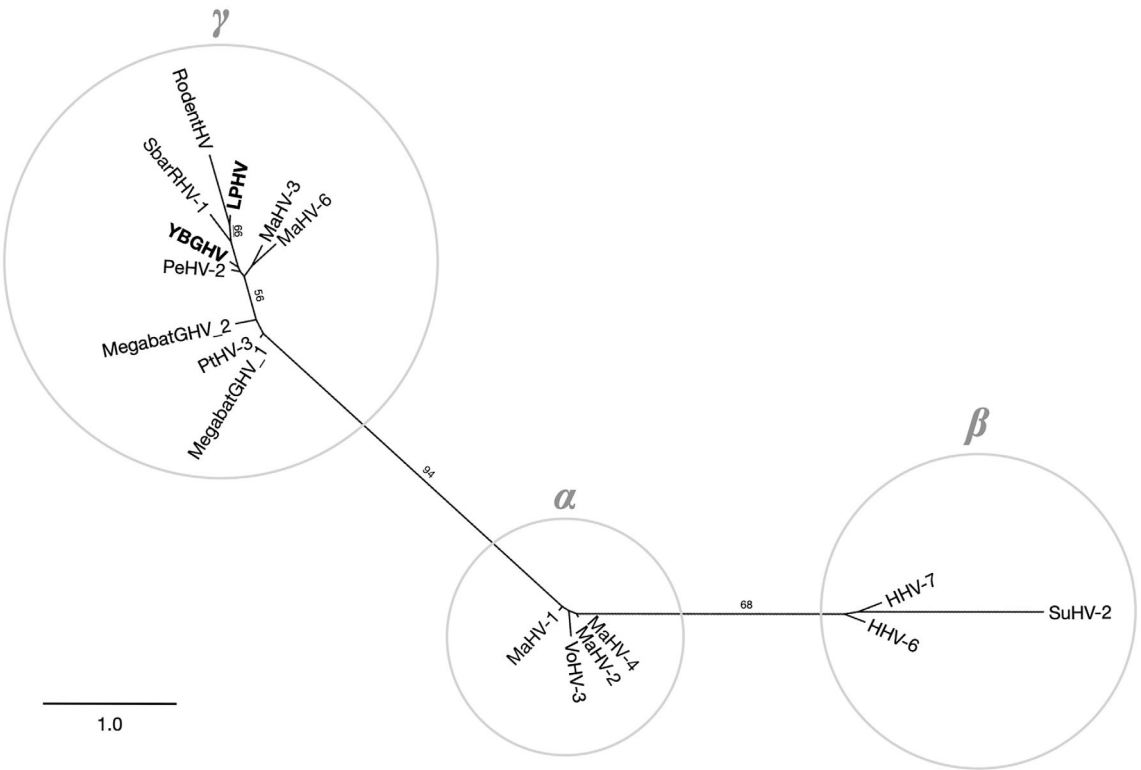

Figure 1. **Maximum-likelihood phylogeny of the deduced amino acid sequences from the UHPCR DPOL region (36 amino acids), showing the inferred relationships between the novel yellow-bellied glider herpesvirus (YBGHV), the Leadbeater's possum herpesvirus (LPHV), their top five BLAST matches, and other representative alpha- (*α*), beta- (*β*) and gammaherpesviruses (*γ*). Bootstrap values (≥50 of 100 iterations) are shown on branches, branch lengths indicate phylogenetic distance, and scale bar shows substitutions per amino acid. The small MaHV-2 and -4 branch has a bootstrap value of 83, which was not possible to clearly indicate in the figure. HHV6, *Human betaherpesvirus 6* (AXS63146); HHV7, *Human betaherpesvirus 7* (AAC40752); MaHV-1, *Macropodid alphaherpesvirus 1* (AMB16995); MaHV-2, *Macropodid alphaherpesvirus 2* (QOD40189); MaHV-3, *Macropodid gammaherpesvirus 3* (ABO61861); MaHV-4, *Macropodid alphaherpesvirus 4* (MT707948); MaHV-6, *Macropodid gammaherpesvirus 6* (BAU98236); MegabatGHV_1, megabat gammaherpesvirus (BBA93921); MegabatGHV_2, megabat gammaherpesvirus (BBA93902); PeHV-2, *Peramelid gammaherpesvirus 2* (UAW09503); PtHV-3, *Pteropodid gammaherpesvirus 3* (UBB58403); RodentHV, rodent herpesvirus (AYU70915); SuHV-2, *Suid betaherpesvirus 2* (ADV78258); SbarRHV-1, *Sus barbatus rhadinovirus 1* (AAO46908); VoHV-3, *Vombatid gammaherpesvirus 3*.[1]

and the Leadbetter's possum samples did not correspond to any highly similar matches in GenBank or the literature.[1, 3, 7–9] The most similar match for each were all from the subfamily *Gammaherpesvirinae*. A phylogenetic tree consisting of the novel sequences, their closest BLASTp matches, and other representative herpesvirus sequences from GenBank and published literature (Figure 1), indicates that two new putative gammaherpesviruses have been identified, herein given the provisional names 'yellow-bellied glider gammaherpesvirus' (YBGHV) and 'Leadbeater's possum gammaherpesvirus' (LPHV). The top BLASTp match was *Peramelid gammaherpesvirus 2* (UAW09503) for both YBGHV (E = 1E-18; 86.4% identity) and LPHV (E = 3E-15; 63.64%), and the hosts of both belong to the *Petauridae* family of marsupials. The *Peramelid gammaherpesvirus 2* sequence was detected in a sample from a free ranging Northern Brown Bandicoot.

Petauridae is one of several families of possums, consisting of three genera and 11 species, including the yellow-bellied glider (*Petaurus australis*) and Leadbeater's possum (*Gymnobelideus leadbeateri*). Yellow-bellied gliders are widely distributed across eastern Australia and are classified as near threatened by the International Union for Conservation of Nature.[14] In contrast, Leadbeater's possum is restricted to old-growth forests of the Central Highlands in Victoria, Australia, and is classified as critically endangered by the International Union for Conservation of Nature.[15] Samples from these species tested in this study were from captive animals in Victoria.

Herpesvirus DNA was detected in a buffy coat sample collected from the yellow-bellied glider in 1996 and stored at −80°C, from which a 133 bp sequence was obtained (Supplementary Material). Herpesvirus DNA from Leadbeater's possums was detected in samples taken postmortem from two (of four) individuals in 2020. Specifically, the liver, lung and spleen of one individual and the spleen (but not liver or lung) of a second individual, each tested positive with 163 bp of quality DPOL sequence was obtained from spleen samples (Supplementary Material). Herpesvirus DNA was not detected in the livers, lungs or spleens of the two other individuals. The UHPCR DPOL amplicons were sequenced from each of the four positive samples. All sequences were similar, but only one was of high quality and used for phylogenetic analysis.

Detection of these novel sequences may be consistent with detection in their natural host, although spill over from other species cannot be excluded until more widespread detection in these or other hosts.

The YBGHV and LPHV are the first herpesviruses detected in any species of possum by molecular methods, and this has enabled the identification of these as gammaherpesviruses. Herpesvirus-like particles have previously been detected by transmission electron microscopy of the intestinal contents from two invasive (non-petaurid) common brush-tail possums (*Trichosurus vulpecula*) in New Zealand.[16] The identification of new species of virus in a near threatened and a critically endangered host has potential conservation significance, and further research is needed to determine the origin of these viruses and whether any clear associations with disease may exist. The short sequences obtained in this study are sufficient for subfamily assignment, but broader genome sequencing is needed to determine their relationships with other members of the *Gammaherpesvirinae*.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.13202/suppinfo.

Appendix S1. Supporting information.

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