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Identification and isolation of a novel herpesvirus in a captive mob of eastern grey kangaroos (*Macropus giganteus*)

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Abstract

A novel herpesvirus was detected in a captive mob of eastern grey kangaroos (*Macropus giganteus*) during diagnostic workup for individuals with ulcerative cloacitis. Virus was initially detected in tissues using a consensus herpesvirus PCR. No viral inclusions or particles had been evident in routine histologic or transmission electron microscopic sections of cloacal lesions. Virus was isolated from samples and transmission electron microscopy of the resulting isolates confirmed that the virus was morphologically consistent with a herpesvirus. Nucleotide sequencing of the PCR product from tissue samples and from the isolates revealed that the virus was in the subfamily *Gammaherpesvirinae* and was distinct from other known herpesviruses. The correlation between the lesions and the novel virus remains unknown. Two herpesviruses, both in the subfamily *Alphaherpesvirinae*, have previously been described in macropods and are known to cause systemic clinical disease. This is the first reported gammaherpesvirus within the order Marsupialia, and may provide valuable information regarding the evolution and phylogeny of this virus family. Based on current herpesvirus nomenclature convention, the authors propose the novel herpesvirus be named Macropodid herpesvirus 3 (MaHV-3).

Keywords: Eastern grey kangaroo; Herpesvirus; Macropod; *Macropus giganteus*; Macropodid herpesvirus 3

1. Introduction

The family *Herpesviridae* are enveloped, double-stranded DNA viruses. Two herpesvirus species have been previously described in Macropodidae (kangaroos

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and wallabies)—Macropodid herpesvirus 1 (MaHV-1) and Macropodid herpesvirus 2 (MaHV-2). Both viruses have been classified in the subfamily *Alphaherpesvirinae* based on genetic sequencing and biological characteristics (Johnson et al., 1985; Johnson and Whalley, 1990; Mahony et al., 1999; Davison et al., 2005).

MaHV-1 was first identified by Finnie et al. (1976) in a captive mob of parma wallabies (*Macropus parma*). The viral infection produced fatal systemic disease resulting in severe clinical signs including pyrexia, respiratory signs, conjunctivitis, and anogenital vesicles (Finnie et al., 1976; Acland, 1981). MaHV-2 was later identified in 1978 in captive grey dorcopsis (*Dorcopsis muelleri luctuosa*) and quokkas (*Setonix brachyurus*) (Callinan and Kefford, 1981). This virus also resulted in fatal systemic disease characterized by conjunctivitis and lesions on the oral and anogenital mucous membranes. Latent infection with an unclassified alphaherpesvirus was described in the eastern grey kangaroo (*M. giganteus*) (Guliani et al., 1999). Herpesvirus infections have also been reported or suspected in other macropod species including tammar wallabies (*M. eugenii*) and western grey kangaroos (*M. fuliginosus*), as well as in two species in the family Potoroidae—brush-tailed rat kangaroos (*Bettongia penicillata*) and rufous rat kangaroos (*Aepyprymnus rufescens*) (Dickson et al., 1980; Callinan and Kefford, 1981; Wilks et al., 1981). In addition to these natural infections, transient experimental infections with MaHV-1 have been produced in the brushtail possum (*Trichosurus vulpecula*) (Zheng et al., 2004). A serologic survey revealed that 23% of wild marsupials tested and 41% of captive marsupials in Australia tested had circulating serum-neutralizing antibodies to a parma wallaby herpesvirus (Webber and Whalley, 1978). However, it is unknown whether the seropositivity reflects exposure to known macropod herpesviruses, previously undescribed herpesviruses, or cross-reaction with antibodies to other antigens unrelated to herpesvirus exposure.

In 2005, a novel herpesvirus was found in a captive mob of eastern grey kangaroos while performing diagnostic workups to determine the etiology for ulcerative cloacitis. No clinical signs or evidence of herpesvirus infection were detected in the mob prior to 2005.

2. Materials and methods

2.1. Animal information

During the sampling period, the captive mob was composed of 45 individuals consisting of 2 adult breeding males, 22 adult females, and 21 joeys less than 3 years of age (9 males, 9 females, and 3 unknown gender). Some of the adult females were captive-bred in the United States, some were captive-bred in Australia, and some were wild-caught rehabilitated animals. The individuals that originated in Australia were imported into the United States between June 1987 and December 1989. Fifteen of the 45 kangaroos in the mob (33.3%; mean age = 8.11 years; median age = 2.17 years) were evaluated for the presence of herpesvirus.

2.2. Sample collection

Samples collected from live animals included whole blood, cloacal swabs, and cloacal biopsies (Table 1). Whole blood was collected from the lateral tail vein and placed in glass EDTA Vacutainer blood tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Cloacal swabs were obtained using sterile cotton-tipped applicators. Cloacal biopsies were obtained using a sterile punch biopsy. Samples collected at necropsy for PCR and viral isolation included 1–5 cm sections of cloacal mucosa, liver, spleen, mammary gland, and lymph node. These samples were placed in either sterile Vacutainer tubes with no additive (Becton, Dickinson and Company, Franklin Lakes, NJ) or in sterile Whirl-Pak bags (Nasco International, Inc., Fort Atkinson, WI), and either submitted fresh on ice packs or stored frozen. Samples for histopathology were placed in 10% neutral buffered formalin. Samples submitted for electron microscopy were placed in Karnovsky's Fixative.

2.3. Histopathology/electron microscopy

Tissues collected at biopsy and necropsy were fixed in 10% neutral buffered formalin, routinely processed, sectioned at 5 μm, and stained with hematoxylin and eosin (HE) for routine light microscopy. For papillomavirus immunohistochemistry, endogenous peroxidase was blocked in deparaffinized and rehydrated sections by immersing sections in hydrogen peroxide

Table 1
Summary of gross lesions, PCR testing, and virus isolation performed on the eastern grey kangaroo mob

Kangaroo #	Sex	Collection date	Age at sampling (years)	Post-mortem?	Mamm. tumor	Cloacal lesions	PCR						Virus isolation			
							B	L	S	MG	LN	C	S	MG	LN	C
1	1.0	22-Mar-06	1.14	N	–	+	–					+				
2	1.0	22-Mar-06	1.16	N	–	–	+					+				
3	0.1	20-Apr-05	1.49	Y	–	+						+				
4	1.0	13-Mar-07	1.60	N	–	–	–					+				
5	1.0	12-Jul-05	1.87	N	–	+	+					+				
6	1.0	12-Jul-05	1.88	N	–	–	+					+				
7	1.0	13-Mar-07	2.03	N	–	–	–					+				
8	1.0	13-Mar-07	2.17	N	–	–	–					+				
5	1.0	22-Mar-06	2.56	N	–	+	–					+				
9	1.0	14-Jul-05	7.67	N	–	–	–					+				
10	1.0	2-Dec-06	10.90	Y	–	–	–		+		+	–				
11	0.1	28-Jan-05	14.40	Y	+	+		–	+							
12	0.1	21-Sep-05	15.05	Y	+	+		–	+	–		+	+			Co
13	0.1	15-Jun-05	∼18	N	+	+						+				
13	0.1	17-Sep-05	∼18	Y	+	+		–	+	+		–	+	+		
14	0.1	21-Dec-05	∼21	Y	+	–	–		+	+		–	+		+	Co
15	0.1	30-Mar-06	∼21.25	Y	+	–			+				Co	–	–	

Virus isolation results marked with "Co" were negative for herpesvirus, but positive for a coronavirus. B = whole blood, L = liver, S = spleen, MG = mammary gland, LN = lymph node, C = cloaca, 1.0 = male, 0.1 = female.

(0.3%) in methanol for 15 min. Slides were rinsed twice in phosphate buffered saline pH 7.6 (BioGenex, San Ramon, CA) for 5 min followed by incubation in a commercially available blocking serum (Power Block Universal Blocking Reagent, Biogenex) for 10 min to prevent non-specific binding. After rinsing in buffer, slides were incubated at room temperature for 30 min with 1:400 polyclonal anti-bovine papillomavirus antibody (BPV-1, Dako, Carpinteria, CA). Negative serum was used on negative control slides. Slides were rinsed in buffer and incubated with the secondary antibody (LSAB2, Dako) at room temperature for 15 min. Following an additional buffer rinse, streptavidin peroxidase (Dako) was applied. After rinsing, the chromagen, 3,3′-diaminobenzidine tetrachloride (DAB; Biogenex), was applied and allowed to develop for 5 min. Slides were rinsed and then counterstained in Mayer's hematoxylin (Biogenex).

For ultrastructural studies, multiple samples containing optimal regions from the cloaca of two kangaroos that were PCR positive for herpesvirus were fixed in Karnovsky's fixative or 2% gluteraldehyde with 2.5% paraformaldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Sections were first cut at 0.35 μm and stained with toluidine blue and basic fuscin for microscopic evaluation of optimal areas to evaluate by electron microscopy and then sectioned at 80–90 nm, stained with uranyl acetate and lead citrate, and examined with a H600 transmission electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA).

2.4. PCR amplification and sequencing

DNA was extracted from frozen tissue samples using the DNeasy Kit (Qiagen, Valencia, CA). Nested PCR amplification of a partial sequence of the DNA-dependent-DNA polymerase gene was performed using previously described methods (VanDevanter et al., 1996). Briefly, the first round of amplification utilized forward primers DFA (5′-GAYTTYGCNA-GYYTNTAYCC-3′, Y = pyrimidine, N = nucleotide) and ILK (5′-TCCTGGACAAGCAGCARNYSG-CNMTNAA-3′, R = purine, M = A or C) and reverse primer PCR amplification utilized forward primer TGV (5′-TGTAACTCGGTGTAYGGNTTYACNGG-NGT-3′) and reverse primer IYG (5′-CACAGAGT-CCGTRTCNCCRTADAT-3′, D = A, G, or T). The mixtures were amplified with an initial denaturation at

94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 60 s, DNA extension at 72 °C for 60 s, and a final extension step at 72 °C for 7 min. Products were resolved on 1% agarose gels and bands of the expected size were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). To obtain additional sequence, an internal reverse primer was designed (primer RooRev TAGTTCTGCCTCGGAGGGTGACGGT) and used in the second round with DFA. Direct sequencing was performed using the Big-Dye Terminator Kit (PerkinElmer, Branchburg, NJ) and analyzed on ABI 377 automated DNA sequencers at the University of Florida Center for Mammalian Genetics DNA Sequencing Facilities. All products were sequenced in both directions. Primer sequences were edited out prior to further analyses.

2.5. Phylogenetic analysis

Sequences were compared with those in GenBank (National Center for Biotechnology Information, Bethesda, MD), EMBL (Cambridge, UK) and the Data Bank of Japan (Mishima, Shiuoka, Japan) using TBLASTX (Altschul et al., 1997). Predicted homologous 153–161 amino acid sequences of herpesviral DNA-dependent-DNA polymerase were aligned using three methods; ClustalW (Thompson et al., 1994), T-Coffee (Notredame et al., 2000), and MUSCLE (Edgar, 2004).

Bayesian analyses of each alignment were performed using Mr.Bayes 3.1 (Ronquist and Huelsenbeck, 2003) with gamma distributed rate variation and a proportion of invariant sites, and mixed amino acid substitution models. The first 10% of 1,000,000 iterations were discarded as a burn in.

Maximum likelihood (ML) analyses of each alignment were performed using PHYLIP (Phylogeny Inference Package, Version 3.66) (Felsenstein, 1989), running each alignment in proml with amino acid substitution models JTT (Jones et al., 1992), PMB (Veerassamy et al., 2003), and PAM (Kosiol and Goldman, 2005) further set with global rearrangements, 5 replications of random input order, less rough, one category of sites, and unrooted. Iguanid herpesvirus 2 (GenBank accession no. AY236869) was designated as the outgroup due to its early divergence from other herpesvirus (Wellehan et al., 2003; McGeoch and Gatherer, 2005). The combination of alignment producing the most likely tree was then used to create data subsets for bootstrap analysis to test the strength of the tree topology (200 resamplings) (Felsenstein, 1985), which was analyzed using the amino acid substitution model producing the most likely tree.

2.6. Virus isolation

A suspension of potoroo kidney cell line (PTK2; ATCC, Manassas, VA, USA) cells was prepared in MEM growth medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 8% fetal bovine serum (FBS) at a concentration of 4 x 10^5 cells/ml in a 25 cm^2 cell culture flask. Consensus herpesvirus PCR was used to confirm that the cell line was free of herpesviruses prior to inoculation (VanDevanter et al., 1996). When monolayers of cells were formed, the PTK2 were infected with a 10% homogenate of the sample tissue prepared in Earl's balanced salt solution and incubated for 2 h, and the inoculum was replaced with 8% FBS cell culture media and incubated in an incubator at 37 °C, 5% CO_2. The cultures were monitored daily for 8 days for the presence of cytopathic effect. Cell cultures were passed to the next flask regardless of whether cytopathic effects were observed or not. Supernatant from the flask was checked for the presence of viral material by electron microscopy and for herpesvirus by PCR.

3. Results

3.1. Clinical disease/gross lesions

Eight of the fifteen kangaroos evaluated (53.3%; 2 males, 6 females) had gross lesions (Table 1). Six of those eight (75.0%; 2 males, 4 females) had cloacal lesions ranging from a small focal mucosal ulcer to chronic fibrinonecrotic cloacitis. Clinical signs associated with the cloacal lesions included overgrooming and licking of the cloacal region, mild cloacal discharge, and bronzing of the hair around the cloaca. Age of affected individuals ranged from 1.14 to 18.0 years (mean age = 8.66 years; median age = 8.14 years). The younger kangaroos exhibited smaller, more acute lesions, while the older individuals had

more severe, chronic lesions with secondary infections.

Five of the eight kangaroos (62.5%; 0 males, 5 females) with gross lesions had firm, irregular masses palpable within the mammary tissue. Age of the female kangaroos with mammary gland masses ranged from 14.40 to 21.25 years (mean age = 17.94 years; median age = 18.0 years). All five individuals with mammary masses died during the sampling period.

Seven of the fifteen kangaroos evaluated (46.7%; 1 male, 6 females) died during the sampling period. Five of the seven were geriatric females with mammary masses. The remaining two kangaroos (3 and 10) died from acute head or neck trauma. No other patterns of gross lesions were detected in those individuals presented for necropsy.

3.2. Histopathology/electron microscopy

Seven of the fifteen kangaroos died and tissues were available for histological evaluation. Antemortem cloacal biopsies were also available from two of these seven animals (12 and 13). Four of seven kangaroos had an ulcerative cloacitis either at biopsy or necropsy. Kangaroos 10, 14, and 15 did not have any cloacal lesions at necropsy. In affected animals, the cloacal mucosal epithelium was markedly hyperplastic with prominent broad, branching rete ridges and parakeratotic hyperkeratosis of the stratum corneum. Within the epithelium there were variable numbers of discrete vesicles containing luminal necrotic cellular debris. Adjacent intact epithelium had both intra and intercellular edema. In a few cases, there were regions of ulceration overlain by fibrin, admixed cell-debris, and superficial bacterial colonization. Bacteria were confined to the superficial crusts and interpreted as secondary colonizers. In a few cases, there were increased numbers of fibroblasts and small blood vessels in the exposed dermis (granulation tissue).

Another significant finding in five kangaroos was simple carcinoma of the mammary gland. With the exception of kangaroo 15, all affected kangaroos had evidence of local lymph node metastasis and kangaroos 12, 13 and 14 had distant (pulmonary and or hepatic) metastasis. No splenic lesions were noted in any of the examined kangaroos. Other histologic findings were considered incidental or age related changes common in this species.

There was no specific immunoreactivity in sections of cloaca using antibodies against *Papillomavirus* sp. in kangaroos 11 and 12.

No viral particles were identified by transmission electron microscopy (TEM) within any cells or in extracellular spaces in sections of cloaca with hyperplastic, vesicular, and ulcerative lesions from kangaroos 3 and 13.

3.3. PCR amplification and sequencing

Initial PCR amplification yielded a 181 base pair product (after editing) from all fifteen kangaroos evaluated. Details of results can be found in Table 1. Nucleotide sequences for all cases were identical. Additional sequence for phylogenetic comparison was obtained from spleen and lymph node samples from kangaroo 10, totaling 472 bp. Sequences were submitted to GenBank under accession number EF467663.

3.4. Phylogenetic analysis

Comparison with other sequences using TBLASTX revealed that this virus is similar to, but distinct from other herpesviruses present in the available databases. The highest score obtained was with *Hylobates leucogenys* rhadinovirus 2 (GenBank accession no. AY465375) . *Hylobates leucogenys* rhadinovirus 2 is in the subfamily *Gammaherpesvirinae*.

Bayesian phylogenetic analysis showed the greatest harmonic mean of estimated marginal likelihoods using the MUSCLE alignment (Fig. 1). The Wag model of amino acid substitution was found to be most probable with a posterior probability of 0.726 (Whelan and Goldman, 2001), and a posterior probability of 0.274 for the Rtrev model (Dimmic et al., 2002). A Bayesian tree using the MUSCLE alignment is shown (Fig. 2).

ML analysis found the most likely tree from the MUSCLE alignment and the PMB model of amino acid substitution. These parameters were used for bootstrap analysis. Bootstrap values from ML analysis are shown on the Bayesian trees. One significant difference of the ML methods used from the Bayesian

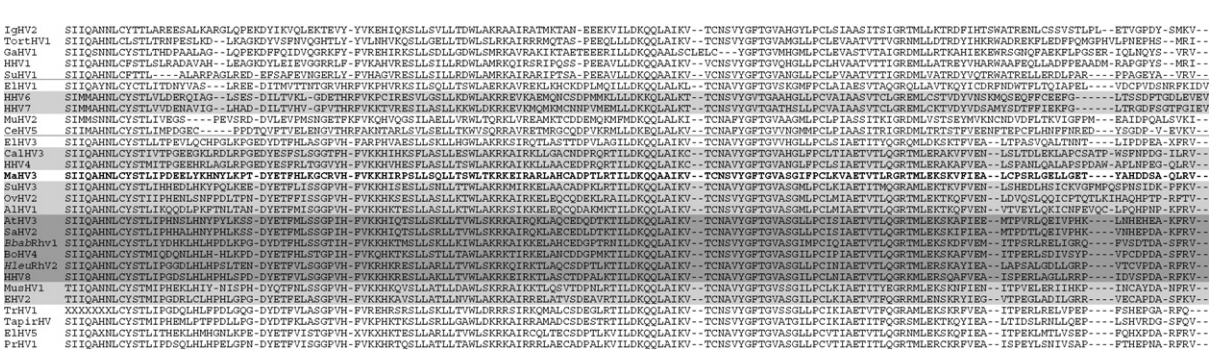

Fig. 1. Alignment of predicted partial herpesviral DNA-dependent-DNA polymerase amino acid sequences created using MUSCLE. Subfamilies are separated by lines, and genera represented by more than one virus are shaded. Macropodid herpesvirus 3 is in bold. Sequences retrieved from GenBank include Alcelaphine HV1 (AlHV1) (AF005370), Ateline HV3 (AtHV3) (AF0834240), *Babyrousa babyrussa* rhadinovirus 1 (*Bbab*RhV1) (AY177146), Bovine HV4 (BoHV4) (AF318573), Callitrichine HV3 (CalHV3) (AF319782), Cercopithecine HV5 (CeHV5) (AY117754), Elephantid HV1 (ElHV1) (AF322977), Elephantid HV3 (ElHV3) (DQ238845), Elephantid herpesvirus 5 (ElHV5) (EF032640), Equid HV2 (EHV2) (U20824), Gallid HV1 (GaHV1) (AF168792), Human HV1 (HHV1) (X14112), Human HV4 (HHV4) (DQ279927), Human HV6 (HHV6) (X83413), Human HV7 (HHV7) (AF037218), Human HV8 (HHV8) (U93872), *Hylobates leucogenys* rhadinovirus 2 (*Hleu*RhV2) (AY465375), Iguanid HV2 (IgHV2) (AY236869), Murid HV2 (MuHV2) (AY728086), Mustelid herpesvirus 1 (MusHV1) (AF376034), Ovine HV2 (OvHV2) (DQ198083), Procavid herpesvirus 1 (PrHV1) (EF032641), Saimiriine HV2 (SaHV2) (AJ410493), Suid HV1 (SuHV1) (BK001744), Suid HV3 (SuHV3) (AF478169), Tapir herpesvirus (TapirHV) (AF141887), Tortoise HV1 (TortHV1) (AB047545), Trichechid herpesvirus 1 (TrHV1) (DQ238847).

methods used is the use of a single rate category for sites, which may decrease accuracy.

3.5. Virus isolation

Samples from four kangaroos were submitted for virus isolation (Table 1). Virus was isolated from three of the four kangaroos and the presence of virus was confirmed by TEM, PCR, and sequencing of amplified product. A virus with morphology consistent with herpesviruses (an enveloped virus around 150 nm in size) was identified by TEM. PCR testing on cell culture and tissue homogenate confirmed the presence of viral DNA in the samples tested. Cytopathic effects seen in culture included cell rounding, cytoplasmic stranding, and cell nucleus enlargement with some cell destruction visible.

A coronavirus was also isolated from three of the kangaroos. Herpesvirus was not isolated from any of the samples that were positive for coronavirus.

4. Discussion

Diagnostic testing to evaluate the cause of an ulcerative cloacitis in a captive mob of eastern grey kangaroos resulted in the discovery of a novel herpesvirus. Based on sequencing of a PCR product, the novel virus is classified in the subfamily *Gammaherpesvirinae*. All previously described herpesviruses in marsupials have been classified in the subfamily *Alphaherpesvirinae*.

The novel herpesvirus does not appear to be associated with the same high degree of mortality associated with MaHV-1 and MaHV-2. Additionally, the clinical signs are not as severe or widespread in the eastern grey kangaroo. The only clinical sign detected in the mob suggestive of herpesvirus infection was the ulcerative cloacitis. However, not all PCR positive animals had cloacitis, and one individual with cloacitis was negative by PCR for herpesvirus in sections of cloaca. Additionally, the presence of virus within the affected cloacal regions could not be demonstrated by histopathology or TEM. Therefore, the potential role of this herpesvirus in the pathogenesis of ulcerative cloacitis remains unclear. In addition, definitive viral cell tropism has yet to be determined. The chronicity of the lesions and the presence of secondary bacterial infections affected the gross and histologic appearance of the lesions.

PCR and virus isolation results revealed the presence of virus DNA and/or viral particles in a

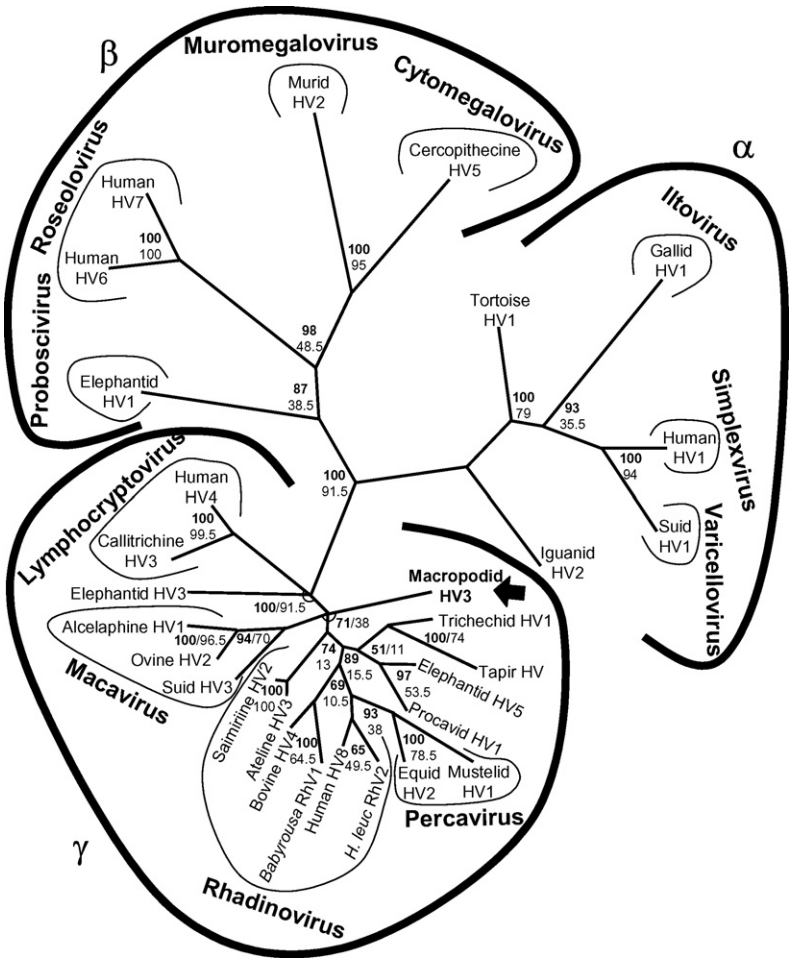

Fig. 2. Bayesian phylogenetic tree of predicted partial herpesviral DNA-dependent-DNA polymerase amino acid sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold, and ML bootstrap values for branchings based on 200 re-samplings are given below. Iguanid HV2 (GenBank accession no. AY236869) was used as the outgroup. Herpesviral genera are delineated by thin brackets, and subfamilies are delineated by thick brackets. Macropodid herpesvirus 3 is bolded and marked by arrows. GenBank accession numbers are given in the legend to Fig. 1.

variety of different tissues, including whole blood. PCR-positive sampling sites included areas with gross lesions (e.g. cloaca, mammary tissue), as well as those with no evidence of gross or histologic abnormalities. The lack of correlation further underscores the uncertainty of this virus' pathogenicity.

The exact origin of the novel herpesvirus and the reason for the acute onset of ulcerative cloacitis remains unknown. The location of the cloacal lesions is suggestive of venereal transmission. However, the exact modes of transmission and the presence of the novel virus in wild populations of eastern grey kangaroos remain unknown. It is possible that advancing age and concurrent disease caused recrudescence of a latent herpesviral infection in a few individuals. Virus particles could then be spread throughout the rest of the mob through direct contact from breeding activity and other interactions. It is possible that the presence of neoplasia in geriatric individuals caused immunosuppression, resulting in a recrudescence of a latent herpesviral infection.

It is unknown whether the novel herpesvirus is related to the seemingly high incidence of mammary neoplasia in the mob. Gammaherpesviruses have been

associated with carcinomas in sealions (Lipscomb et al., 2000), but further research will be needed to assess the possible association in kangaroos.

The herpesvirus sequence in this study is from a conserved region, and these differences together with host species are significant enough to differentiate this from other known herpesvirus species. In another study, analysis of a smaller portion of this region in five strains of human herpesvirus-2, 17 strains of human herpesvirus-6, and five strains of human herpesvirus-7 were sequenced. Only single base variations were seen within a given species, which did not result in alteration of the amino acid sequence (VanDevanter et al., 1996). Over this shorter region, the novel virus in this study shared identity with only 34 of 55 amino acids when compared to the most homologous sequence, indicating this virus is representative of a new species.

Current naming conventions for herpesviruses use host family and order of virus discovery (Davison et al., 2005). There are two previously described herpesviruses from members of the Macropodidae, Macropodid herpesvirus 1 (MaHV-1), and Macropodid herpesvirus 2 (MaHV-2), both of which are in the subfamily *Alphaherpesvirinae*. The phylogenetic tree demonstrates that the virus in this study significantly clusters with other herpesviruses in the subfamily *Gammaherpesvirinae* (Fig. 2). Based on naming conventions, this virus should be named Macropodid herpesvirus 3 (MaHV-3). The topology is generally consistent with that of phylogenetic trees generated from other data sets (Wellehan et al., 2003; McGeoch and Gatherer, 2005).

Comparative sequence analysis of the herpesviruses of diverse host species should contribute to a further understanding of viral phylogeny and the evolution of this important class of viruses. Previous phylogenetic analyses suggest that many elements in the branching patterns of *Herpesviridae* are congruent with branching patterns for the corresponding host species (Jackson, 2005, McGeoch et al., 2006). The branching of the alphaherpesviruses from the beta- and gammaherpesviruses has been estimated to have occurred approximately 374–413 million years ago, and the branching of the betaherpesviruses from the gammaherpesviruses approximately 331–351 million years ago (McGeoch and Gatherer, 2005). Within the gammaherpesviruses, the divergence of the *Rhadinovirus*/*Percavirus* lineages has been estimated to have occurred 70–73 million years ago (McGeoch et al., 2005). Looking at host species, the divergence between marsupials and placental mammals has been estimated to have occurred 193–186 million years ago (van Rheede et al., 2006), which would occur temporally after the estimated beta/gamma split but before *Rhadinovirus*/*Percavirus* divergence. This is in agreement with the phylogenetic analysis, and supports coevolution.

The availability of more complete data sets for comparison results in greater phylogenetic resolution (Flynn et al., 2005). The herpesvirus polymerase is the gene for which the most comparative sequence from other herpesviral species is available. Additionally, when looking at evolutionary relationships of more distantly related organisms, the continued accrual of mutations resulting in homoplasy can diminish the ability to correctly resolve phylogeny, making a rapidly mutating gene a poor choice. Genes for which there is strong negative selection will have fewer nucleotides with a history of multiple changes, making them a better choice for resolving phylogeny over greater distances. Genes that are critical for basic organismal functions and are not under heavy immune selection are often highly conserved. Therefore, viral polymerases are usually good choices for long-range phylogeny (Attoui et al., 2002; Gonzalez et al., 2003; Knopf, 1998).

It has recently been proposed to split the genus *Rhadinovirus* into three genera; *Macavirus* containing the malignant catarrhal fever viruses, *Percavirus* containing perissodactylid and carnivore gammaherpesviruses, and *Rhadinovirus* containing the remaining viruses from the old genus (McGeoch et al., 2006). While the analysis in this study supports clear divergence of the proposed *Macavirus* from other genera, the division between *Rhadinovirus* and *Percavirus* is not as clear, and the new *Rhadinovirus* appears to be paraphyletic in this analysis. One possible explanation for the differences in this analysis from previous analyses is the inclusion of MaHV-3 and Afrotherian gammaherpesviruses in this analysis. MaHV-3 clusters with the *Rhadinovirus*/*Percavirus*/*Macavirus* lineages, but does not cluster with any one genus, and may be a distinct lineage. Other possible explanations for the differences seen are the assessment of multiple alignment algorithms and substitu-

tion models for optimality in this study. Both nucleotide sequence alignment and amino acid substitution models may have significant effects on results (Morrison and Ellis, 1997, Whelan and Goldman, 2001). Finally, this analysis is of a smaller data set from a single gene which may have resulted in error.

The other marsupial herpesviruses, MaHV-1 and MaHV-2, are members of the genus *Simplexvirus* in the subfamily *Alphaherpesvirinae* (Davison et al., 2005). They are closely related to Eutherian viruses in the genus *Simplexvirus*, and appear to be an example of host switching rather than coevolution (Mahony et al., 1999; Lee and Smith, 1999). *Alphaherpesvirinae* are often capable of infecting a wider range of host cells than beta- and gammaherpesviruses (Davison et al., 2005), and this may make a host switch more feasible. MaHV-3 may represent a lineage that has evolved in marsupials, may have arisen very close to the basal branching point of the gammaherpesviruses, and may provide insight into the genetic structure of the ancestral herpesviruses. Further sequence and analysis of this virus is indicated to clarify.

5. Conclusion

Macropodid herpesvirus 3 (MaHV-3) is a novel gammaherpesvirus discovered in a captive mob of eastern grey kangaroos. Although the clinical signs observed in the mob could not be definitively linked to the novel virus, MaHV-3 in the eastern grey kangaroo does not appear to result in the same severity of clinical signs associated with the previously described alphaherpesviruses in marsupials. This is consistent with a longer established host-virus coevolutionary relationship as seen in other herpesviruses, whereas the often-fatal disease seen with MaHV-1 and MaHV-2 may suggest a more recent host switch. Comparison of sequencing data of a PCR product obtained from the virus to sequencing data from other known herpesviruses classifies the novel virus in the subfamily *Gammaherpesvirinae*. However, the novel virus could not easily be placed into any of the four proposed genera within that subfamily. Being the first gammaherpesvirus found in marsupials, MaHV-3 may provide valuable information into the evolution and subsequent phylogenetic classification of herpesviruses.

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