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ISOLATION AND CHARACTERIZATION OF A NOVEL HERPESVIRUS FROM A FREE-RANGING EASTERN GREY KANGAROO (*MACROPUS GIGANTEUS*)

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ABSTRACT: We isolated a macropodid herpesvirus from a free-ranging eastern grey kangaroo (*Macropus giganteous*) displaying clinical signs of respiratory disease and possibly neurologic disease. Sequence analysis of the herpesvirus glycoprotein G (gG) and glycoprotein B (gB) genes revealed that the virus was an alphaherpesvirus most closely related to *macropodid herpesvirus 2* (MaHV-2) with 82.7% gG and 94.6% gB amino acid sequence identity. Serologic analyses showed similar cross-neutralization patterns to those of MaHV-2. The two viruses had different growth characteristics in cell culture. Most notably, this virus formed significantly larger plaques and extensive syncytia when compared with MaHV-2. No syncytia were observed for MaHV-2. Restriction endonuclease analysis of whole viral genomes demonstrated distinct restriction endonuclease cleavage patterns for all three macropodid herpesviruses. These studies suggest that a distinct macropodid alphaherpesvirus may be capable of infecting and causing disease in eastern grey kangaroos.

Key words: Alphaherpesvirus, eastern grey kangaroo, herpes, herpesvirus, macropod, MaHV, marsupial, syncytia.

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INTRODUCTION

Members of the family *Herpesviridae* are enveloped, double-stranded, DNA viruses that cause morbidity and mortality in a wide range of species and are classified into three subfamilies, the *Alpha-*, *Beta-*, and *Gammaherpesvirinae* (Pellet and Roizman, 2007). In Australian marsupials, electron microscopy and serologic surveys have detected evidence of herpesvirus infection in possums (*Trichosurus vulpecula*), dasyurids (*Phascogale tapoatafa* and *Antechinus stuartii*), and wombats (*Vombatus ursinus*), but no sequence data from these viruses have been reported (Webber and Whalley, 1978; Barker et al., 1981; Rothwell et al., 1988; Rice and Wilks, 1996). Most recently, two novel gammaherpesviruses (*phascolarctid herpesviruses 1* and 2, PhaHV-1 and PhaHV-2) have been detected in wild koalas (*Phascolarctos cinereus*) by partial sequencing of the conserved region of the DNA polymerase gene (Vaz et al., 2011, 2012). In Macropodidae (kangaroos and wallabies), three distinct herpesviruses have been identified. These include two alphaherpesviruses (*macropodid herpesviruses 1* and *2*, MaHV-1 and MaHV-2; Johnson et al., 1985) and a gammaherpesvirus (*macropodid herpesvirus 3*, MaHV-3; Smith et al., 2008; Wilcox et al., 2011). In captive populations, infection with MaHV-1 or MaHV-2 can lead to fatal systemic disease and severe clinical signs, including conjunctivitis, respiratory signs, pyrexia, and vesicular anogenital lesions (Webber and Whalley, 1978). However, the clinical significance of MaHV-1 and -2 in wild populations is unknown. Disease has not been definitively associated with MaHV-3 but may include ulcerative cloacitis and respiratory disease (Smith et al., 2008; Wilcox et al., 2011).

The two macropodid alphaherpesviruses were originally differentiated by restriction endonuclease cleavage analysis of whole viral genomes (using *Pvu*II, *Bgl*II, and *Eco*RI) and serologic analyses (Johnson et. al., 1985; Webber and Whalley, 1978). Little is known about the

genomic sequences of the macropodid herpesviruses, although sequence data are available for some genes, including those encoding herpesvirus glycoprotein G (gG) and glycoprotein B (gB) genes of MaHV-1 and MaHV-2. *Macropodid herpesvirus 1* and MaHV-2 gB sequences share 82.3% amino acid identity. Interestingly, prior phylogenetic analyses of these gB sequences has placed these two macropodid alphaherpesviruses in a cluster with primate alphaherpesviruses and particularly close to *human herpesvirus 1* (HHV-1) and HHV-2 (Mahony et al., 1999). We report the isolation and characterization of a novel macropodid herpesvirus from a free-ranging eastern grey kangaroo (EGK) that was showing clinical signs of respiratory and, possibly, neurologic disease.

MATERIALS AND METHODS

Viruses and cells

We collected a nasal swab from a free-ranging EGK presenting with rhinitis, conjunctivitis, and tail twitching in September 2009 in western Victoria, Australia, and the sample was used to inoculate subconfluent monolayers of wallaby JU56 fibroblast cells (Uren et al., 1966). The captured animal was one of four animals showing similar clinical signs. The animals were part of a mixed population of free-ranging and reintroduced hand-reared macropods. The MaHV-1 and MaHV-2 isolates used in this study were kindly supplied by Timothy Mahony (Queensland Department of Primary Industries and Fisheries, Australia). All virus stocks, including the unknown virus (designated V3115), were propagated in JU56 cells in a closed culture system at 37 C in Dulbecco's minimal essential medium (DMEM, Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 7.5% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), 10 mM HEPES pH 7.6, and 50 μg gentamicin/mL (Sigma-Aldrich). When performing viral characterization studies, cell cultures were maintained in a 5% CO_2 culture system in DMEM supplemented with 4% (v/v) FBS, 10 mM HEPES pH 7.6, and 50 μg gentamicin/mL.

Cell culture characterization of virus

Polyclonal antibodies for use in serum-virus cross-neutralization assays were generated by the hyperimmunization of rats with MaHV-1, MaHV-2, or V3115 cultured in JU56 cells. Virus culture supernatants were clarified by centrifugation (1,500 × G for 10 min, 3,500 × G for 10 min), and the virus in the supernatant was pelleted (17,000 × G for 60 min) before resuspension in phosphate-buffered saline. Freund's complete adjuvant was added to prepare homogenized emulsions for primary immunization of two rats for each virus preparation. Serum was collected after boosting with similarly prepared virus in Freund's incomplete adjuvant.

Serum-virus cross-neutralization assays were performed using hyperimmune rat serum against each virus. The assays were performed as described (Warner et al., 2001) using JU56 cells. Wells were examined after 4 days incubation, and the reciprocal of the highest dilution of serum that neutralized 50% of virus-infected cultures was recorded as the endpoint calculated using the Karber formula (Karber, 1931).

The capacity of each macropodid herpesvirus to spread from cell to cell was assessed by measuring the size of viral plaques in infected JU56 cells cultured under a 1% methylcellulose (Sigma-Aldrich) overlay medium. Between 14 and 30 plaques per virus were photographed daily for 4 days postinoculation. Plaque sizes were measured using ImageJ (Abramoff et al., 2004). The mean plaque areas induced by each of the viruses at each time point were compared using one-way analysis of variance (ANOVA) followed by post hoc comparisons using Tukey's honest significant difference (HSD) test. $P<0.05$ was considered statistically significant.

To examine differences in plaque morphology, glass coverslip cultures of JU56 cells were infected with each virus and collected daily for 3 days postinoculation and stained with Diff Quik (Lab Aids Pty Ltd, North Narrabeen, New South Wales, Australia). Virus-infected cultures were compared with an uninfected control coverslip culture. The proportion of plaques that showed syncytia formation 3 days after infection was calculated for each virus, and differences were compared using Fisher's exact test. $P<0.05$ was considered significant.

The growth characteristics of the three viruses were assessed by generating multistep growth curves. Viruses were inoculated onto subconfluent JU56 cells in triplicate, and the cell and supernatant fractions (SNF) were harvested separately at 24-hr time points for 5 days and stored at −70 C. The SNF and cell-associated virus fractions were subsequently titrated on JU56 cells to determine the median tissue culture infective dose (Flint et al., 2009). Viral titers at each time point were compared

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using one-way ANOVA followed by Tukey's HSD test. $P<0.05$ was considered significant.

Genetic characterization of virus

Viral DNA was extracted (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany) from JU56 cells infected with V3115 and used as template in PCR-containing primers targeting the gG (Fwd 5′GCGGGGCTATATATTGGCTTG, Rev 5′TAGACATGAGACATGTGGCC) and gB (Fwd 5′ATGACCACACATTCCCCAC-CAAA, Rev 5′TTATGTTTCGTCGTCGGTTT-CATTTA) genes of MaHV-2. Each PCR was incubated through 35 cycles of 94 C for 30 sec, 55 C for 30 sec, and 68 C for 1.5 min using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). Each PCR product was purified (Gel Extraction Kit, Qiagen) and sequenced (Big Dye Terminator version 3.1, ABI PRISM, Applied Biosystems, Carlsbad, California, USA) to obtain full sequence coverage of both strands of each PCR product, without the 5′ and 3′ primer regions. Sequence comparisons of the predicted amino acid sequences of the amplified gG and gB genes were performed using ClustalW (Larkin et al., 2007). Glycoprotein sequences were also analyzed using the bioinformatics prediction algorithms TMHMM Server v. 2.0 (Krogh et al., 2001) and SignalP 3.0 (Bendtsen et al., 2004).

Restriction endonuclease cleavage patterns were determined with viral DNA extracted from JU56 cells infected with each virus (Pignatti et al., 1979). Viral DNA was digested for 4 hr at 37 C using the restriction endonucleases *Pvu*II, *Bgl*II, and *Eco*RI. Fragments were then separated by electrophoresis in a 0.8% agarose gel and stained with ethidium bromide.

RESULTS

Cell culture characterization

After inoculation of JU56 cells with the nasal swab material, a few foci of rounded refractile cells and a focal degenerative type of cytopathic effect consistent with herpesvirus infection were observed after 48 hr. Neutralization results were considered significant if a four-fold difference was observed. Polyclonal antibodies against MaHV-1 did not neutralize MaHV-2 or V3115 at the dilutions tested (Table 1), although antiserum against MaHV-2 neutralized MaHV-1 at a titer of 10. Antibodies against V3115 showed similar cross-neutralization patterns to those raised against MaHV-2, although they were not able to neutralize MaHV-1 at the dilutions tested (Table 1).

TABLE 1. Antigenic relationships between three macropodid alphaherpesviruses in cross-neutralization assays in JU56 cells.[a]

Virus	Neutralization titer[b] of antisera against		
	MaHV-1	MaHV-2	V3115
MaHV-1	50	10	<10
MaHV-2	<10	50	30
V3115	<10	20	20

[a] MaHV = macropodid alphaherpesvirus; V3115 = unknown virus.

[b] Reciprocal of the highest dilution of serum that neutralized 50% of 100 tissue culture infectious dose of virus.

Both V3115 and MaHV-1 formed significantly larger plaques than MaHV-2 at all time points when grown under the methylcellulose overlay ($P<0.001$; Fig. 1A). The largest difference was observed 2 days after infection, when the mean plaque size induced by MaHV-2 was 69.1% smaller than those induced by V3115 (data not shown). No significant difference was observed at any time point between the size of plaques formed by V3115 and MaHV-1 (Fig. 1A). Visual examination of plaque morphology at 3 days postinfection revealed formation of syncytia in plaques induced by V3115 (Fig. 1B) and MaHV-1 (Fig. 1C), but not in plaques induced by MaHV-2 (Fig. 1D). Significantly more plaques contained syncytia in the coverslip cell cultures infected with V3115 than in those infected with MaHV-1 and MaHV-2 (96.7, 34.2, and 0%, respectively, $P<0.001$).

Multistep growth curves for V3115, MaHV-1, and MaHV-2 are shown in Figure 2. V3115 grew to lower titers than either MaHV-1 or MaHV-2 in each of the culture fractions tested (total, SNF-only, and cell-associated). The proportion of infectious virus in the cell-associated fraction was consistently higher in the V3115-infected cell cultures than in cell cultures infected with MaHV-1 or MaHV-2. This difference was significant 3 days after inoculation ($P=0.013$ and $P=0.001$,

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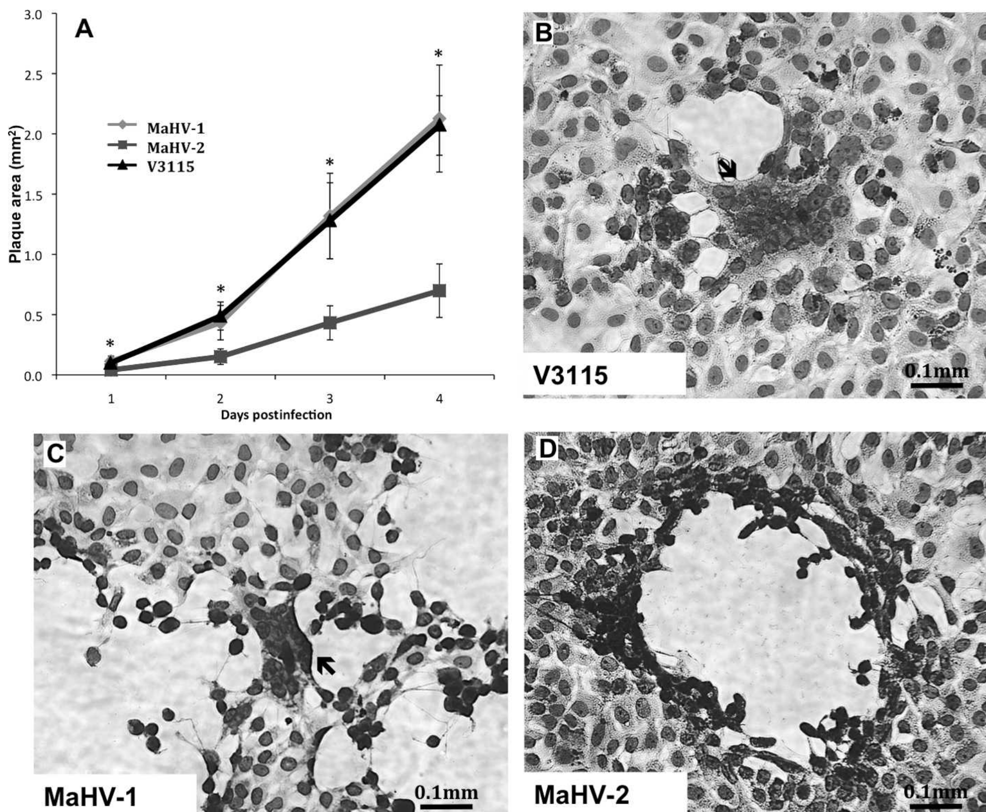

FIGURE 1. Comparison of plaque size and morphology in cell cultures infected with three macropodid alphaherpesviruses (MaHVs): MaHV-1, MaHV-2, and V3115 (unknown virus). (A) Plaque area in JU56 cells was measured using ImageJ over 4 days (with standard deviation bars). Asterisks (*) indicate a significant difference between MaHV-2 and both MaHV-1 and V3115. Photomicrographs of Diff Quik–stained infected JU56 cells at day 3 postinfection showing syncytia (arrows) in cultures infected with (B) V3115 and (C) MaHV-1. (D) Syncytia were not observed in MaHV-2 cultures at any time point.

respectively), when the proportion of virus in the cell fraction was 0.600±0.001 (mean±SD) for V3115, 0.530±0.028 for MaHV-1, and 0.480±0.021 for MaHV-2.

Genetic characterization

Products were amplified successfully from V3115 using primers targeting gG and gB of MaHV-2. No products were amplified using MaHV-1 primers (data not shown). Comparison of the predicted amino acid sequences of the V3115 gG and gB genes showed that they were distinct from those of MaHV-1 (58.5% and 82.4% amino acid sequence identity, respectively) and MaHV-2 (82.7% and 94.6% amino acid sequence identity, respectively; Fig. 3A, B). Key features associated with the predicted tertiary structure of the different gG and gB amino acid sequences were examined. Comparison of the gG sequences (Fig. 3A) identified a transmembrane domain in the C-terminal region of both V3115 and MaHV-2, but no such domain in MaHV-1. In gB (Fig. 3B) the transmembrane helix was conserved between the three viruses, but variation was identified in the number and pattern of distribution of cysteine residues and putative *N*-linked glycosylation sites in both gG and gB of MaHV-2 and V3115. Distinct restriction endonuclease cleavage patterns were observed between all three viruses for all three endonucleases (*Pvu*II, *Bgl*II, and *Eco*RI; Fig. 4). In particular, V3115 had a profile different from that of MaHV-2. The MaHV-1 and -2 profiles

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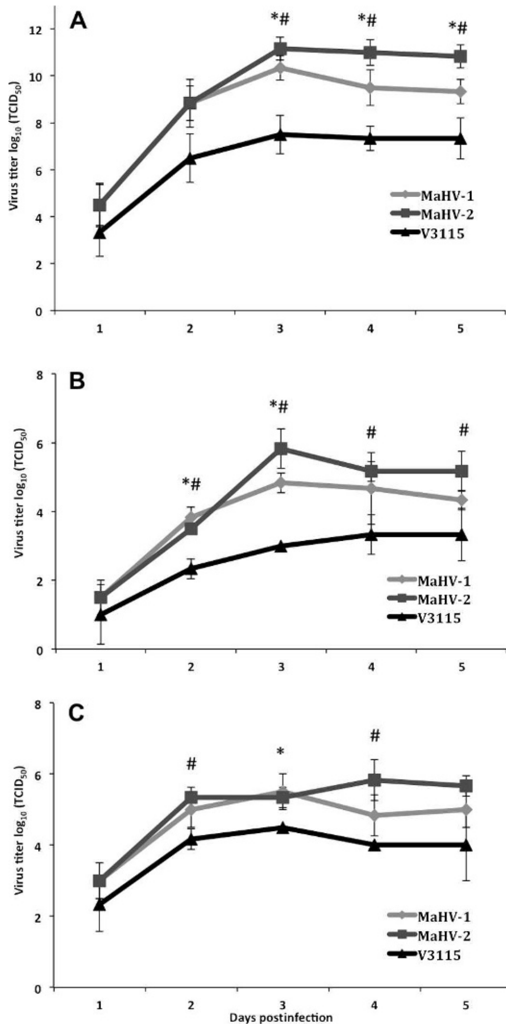

FIGURE 2. Multistep growth curves of three macropodid alphaherpesviruses (MaHVs)—MaHV-1, MaHV-2, and V3115 (unknown virus)—in JU56 cells (with standard deviation bars). Asterisks (*) indicate a significant difference ($P \leq 0.05$) between the titers of V3115 and MaHV-1. Hashes (#) indicate a significant difference between the titers of V3115 and MaHV-2. (A) Total viral titers (both supernatant and cell fractions). (B) Viral titers in the supernatant-only fractions. (C) Viral titers in the cell-associated fractions.

were consistent with those reported previously (Johnson et al., 1985).

DISCUSSION

The alphaherpesvirus examined in this study is the first herpesvirus to be isolated from a free-ranging macropod, albeit from a mixed population; the other macropodid herpesviruses having been isolated from captive animals (Callinan and Kefford, 1981; Smith et al., 2008). In vitro characterization of the virus revealed substantial differences between this virus and the known macropodid herpesviruses. The growth characteristics of V3115 were more similar to those of MaHV-1, whereas genetically and serologically, V3115 was more similar to MaHV-2. Importantly, whole genome restriction endonuclease cleavage analysis showed that V3115 was distinct from both MaHV-1 and MaHV-2. This situation is not unique within the herpesvirus family. Of particular note is the relationship between equine herpesvirus-1 (EHV-1), EHV-4, and EHV-9. These viruses cannot be differentiated on the basis of serum-virus cross-neutralization, have high gB amino acid sequence identity (89.6% between EHV-1 and EHV-4; 97% between EHV-1 and EHV-9), and yet differ markedly in their behavior, as well as in their whole genome restriction endonuclease cleavage patterns (Studdert et al., 1981; Fukushi et al., 1997; Telford et al., 1998). The unique banding profile observed for V3115 is consistent with this isolate being a distinct and novel herpesvirus. For these reasons, we propose that the new virus be tentatively designated *macropodid herpesvirus 4* (MaHV-4), following current conventions for herpesvirus nomenclature.

The V3115 isolate showed an enhanced capacity to spread from cell to cell and form syncytia compared with MaHV-2, with plaques three times larger in area at days 2 to 4, and 96.7% of plaques forming syncytia (none observed in MaHV-2–induced plaques; Fig. 1). Additionally, a higher proportion of virus was cell-associated in V3115-infected cell cultures than in cultures infected with MaHV-2 or MaHV-1 (Fig. 2). Adaptation to cell culture might be responsible for inducing some changes in the in vitro growth characteristics of viruses, particularly in the titer of virus produced (Harrison et al., 1968), and this

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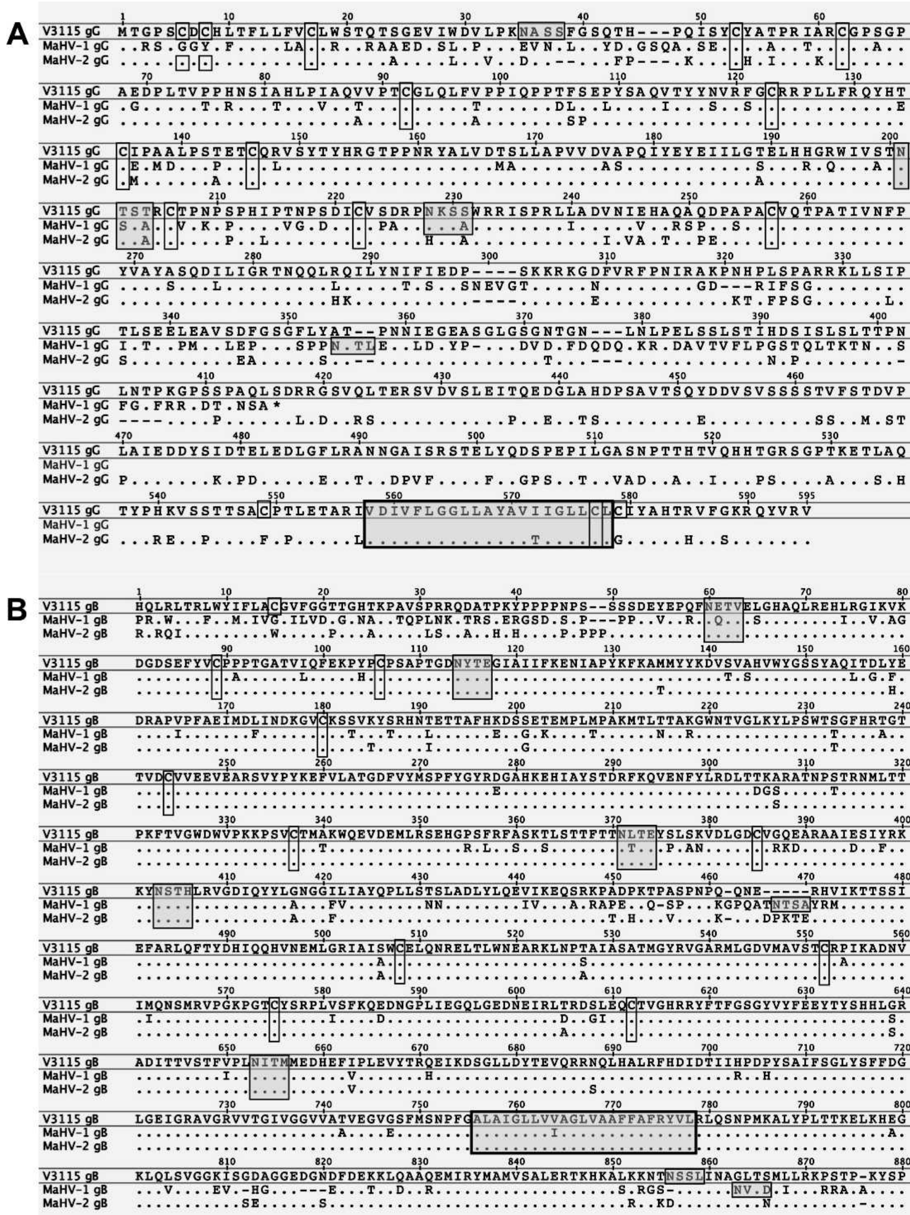

FIGURE 3. Comparison of predicted amino acid sequences from macropodid alphaherpesviruses (MaHVs) MaHV-1 and MaHV-2 to those of V3115 (unknown virus) (MaHV-1, AY048539.1 [herpesvirus glycoprotein G (gG)] and AAD11960.1 [herpesvirus glycoprotein B (gB)]; MaHV-2, AY048540.1 [gG] and AF061755.1 [gB]; V3115, JX397939 [gG] and JX397938 gB]). Dots (.) indicate identity, and dashes (-) indicate gaps or unavailable sequence. Bold shaded boxes indicate transmembrane domains; smaller shaded boxes indicate predicted *N*-linked glycosylation sites, and unshaded boxes indicate cysteine residues. (A) Glycoprotein G amino acid sequence alignment. (B) Glycoprotein B amino acid sequence alignment.

could account for some of the differences reported here (MaHV-1 and MaHV-2 were used at passage 22, whereas V3115 was used at passage 5). However, the genetic stability of alphaherpesviruses (Karlin et al., 1994), combined with the striking differences in cell-to-cell spread and capacity to form syncytia, suggest that these pheno-

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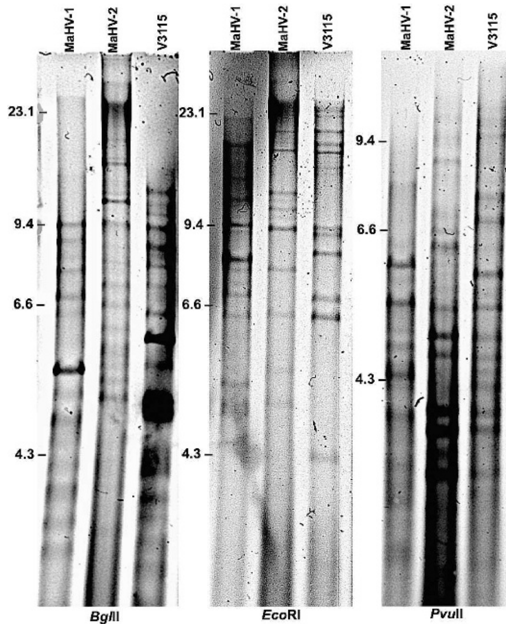

FIGURE 4. Restriction endonuclease analysis of whole viral genomic DNA from macropodid alphaherpesviruses (MaHVs) MaHV-1, MaHV-2, and V3115 (unknown virus) using restriction endonucleases *Bgl*II, *Eco*RI, and *Pvu*II.

typic differences might be due to fundamental differences that affect growth characteristics of V3115, rather than cell culture adaptation. This is supported by restriction endonuclease cleavage analysis of the whole viral genome, which detected differences in banding profiles among the three viruses. The three restriction enzymes chosen were those used to originally differentiate MaHV-1 and -2, and the patterns observed in this study are consistent with those from earlier studies (Johnson et. al., 1985).

In this study, the genetic analyses of the novel and the known macropodid alphaherpesviruses focused on the gG and gB genes. Glycoprotein B is a highly conserved herpesvirus glycoprotein and is one of the more commonly sequenced herpesvirus genes. These features make this gene well suited to genetic comparisons of herpesvirus species (Pellet and Roizman, 2007). Glycoprotein G can also be useful for the serologic differentiation of alphaherpesviruses because it is highly variable (Hartley et al., 1999). Analyses of the V3115 gG and gB genes showed that they had predicted amino acid sequence identities with MaHV-2 of 82.7% and 94.6%, respectively, indicating that they are closely related. Some variation in the number and pattern of distribution of *N*-linked glycosylation sites and cysteine residues were observed, although the biological significance of these differences is unknown. The roles of gG and gB in macropodid herpesviruses have not been studied, but in other alphaherpesviruses these proteins have roles in immune evasion (Bryant et al., 2003; Devlin et al., 2010) and virus entry/cell-to-cell spread (Bzik et al., 1984; Knipe, 2007), respectively. Given the considerable differences between V3115 and MaHV-2 in cell-to-cell spread and formation of syncytia observed in this study, future studies to investigate the relationship between the different macropodid herpesvirus gB genotypes and their associated in vitro phenotypes would be useful.

This is the first isolation of a herpesvirus from a free-ranging macropod with clinical signs of disease. Serologic studies have shown that a number of different free-ranging Australian marsupial species, including EGKs, possess neutralizing antibodies against MaHV-1 and MaHV-2, but the clinical significance of infection with herpesviruses in these free-ranging populations is not known (Webber and Whalley, 1978; Barker et al., 1981; Rothwell et al., 1988). Because these earlier reports were based on detection of antibody and not isolation of the viruses that induced them, it is not known exactly which herpesviruses were present because cross-neutralization has been reported frequently between herpesviruses from related and distant hosts (Wilks et al., 1981). Polyclonal antibodies raised against V3115 had lower homologous neutralizing titers than those generated by MaHV-1 and -2 but still cross-neutralized MaHV-2 virus to a similar level (Table 1), suggesting that previous serologic prevalence

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studies would not have differentiated these two viruses. Detection and isolation of this herpesvirus from a nasal swab of a diseased animal in the midst of an outbreak of respiratory disease suggests that this virus could be associated with the disease, but without performing animal infection trials, it is difficult to demonstrate causation. The clinical signs of respiratory disease observed in the outbreak were similar to those described in alphaherpesvirus infections of captive macropod populations (Finnie et al., 1976), although the tail-twitching observed in the free-ranging animals has not been reported. Tail twitching might indicate neurologic disease, which has been widely reported with herpesvirus infections in other species (Whitley, 2006).

Our results contribute to understanding herpesviruses in free-ranging macropod populations. Previous herpesvirus prevalence studies utilized serologic methods to study free-ranging marsupial populations (Webber and Whalley, 1978). Our results, as well as other recent reports of novel herpesvirus infections in free-ranging marsupials (Vaz et al., 2011, 2012; Wilcox et al., 2011), highlight the importance of applying molecular biological technologies to better detect and differentiate herpesviruses in wild populations.

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