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Short communication

Domestic sheep and bighorn sheep carry distinct gammaherpesviruses belonging to the genus *Macavirus*

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ABSTRACT

The genus *Macavirus* of the subfamily *Gammaherpesvirinae* comprises two genetically distinct lineages of lymphotropic viruses. One of these lineages includes viruses that can cause malignant catarrhal fever (MCF), which are known as MCF viruses (MCFV). All MCFVs are genetically and antigenically related but carried by different hosts. In this study, we report the recognition of new MCFV carried by bighorn sheep. The virus was first identified in a bighorn sheep from Banff National Park, Alberta, Canada. Analysis of a conserved region of the viral DNA polymerase gene of the virus carried by this bighorn sheep showed 85.88% nucleotide identity to the MCFV carried by domestic sheep, ovine herpesvirus 2 (OvHV-2). Further investigation of bighorn samples obtained from animals in the US and Canada showed 98.87–100% identity to the DNA polymerase sequence of the first bighorn in the study. Phylogenetic analysis indicated that the MCFV carried by bighorn sheep is closely related but distinct from OvHV-2. Epidemiological and virulence features of the newly recognized MCFV are still unknown and warrant further investigation. Considering the current nomenclature for MCFVs, we suggest a tentative designation of ovine herpesvirus-3 (OvHV-3) for this newly identified bighorn sheep MCFV.

The genus *Macavirus* in the subfamily *Gammaherpesvirinae* includes two lineages of lymphotropic viruses. Viruses in one of the lineages can cause a severe disease known as malignant catarrhal fever (MCF), and are, therefore, known as MCF viruses (MCFV). The creation of the *Macavirus* genus was proposed by the Herpesvirus Study Group in 2005 and is currently recognized by the International Committee on Taxonomy of Viruses (Davison et al., 2009; McGeoch et al., 2006). Prior to this classification, MCFVs were identified as ruminant rhadinoviruses type 1, and designated as members of the MCFV group (Li et al., 2005b). The MCF group includes an expanding number of closely related viruses carried by several different ruminant species. Malignant catarrhal fever viruses are antigenically and genetically similar and have been characterized by the presence of the 15-A epitope in glycoprotein B and a high level of identity in conserved regions of the DNA polymerase gene (Li et al., 2001a). Currently, five MCFVs are listed in the genus *Macavirus*: *Ovine herpesvirus 2*(OvHV-2), *Alcelaphine herpesvirus 1*(AlHV-1), *Alcelaphine herpesvirus 2*(AlHV-2), *Caprine herpesvirus 2*(CpHV-2), and *Hippotragine herpesvirus 1*(HiHV-1) (Davison et al., 2009). However, several other similar MCFVs have been recognized over the years and named based on their reservoir hosts (Klieforth et al., 2002; Li et al., 2000, 2001, 2003, 2005, 2013). Malignant catarrhal fever viruses have been found as persistent and usually asymptomatic infections in adapted hosts, usually ruminants in the subfamilies *Alcelaphinae*, *Hippotraginae* and *Caprinae*. Disease typically occurs when an MCFV is transmitted from an adapted host to one of several ungulate species that are non-adapted to the virus. Malignant

Abbreviations: MCF, malignant catarrhal fever; MCFV, malignant catarrhal fever virus; OvHV-2, ovine herpesvirus 2; OvHV-3, ovine herpesvirus-3; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; cELISA, competitive enzyme-linked immunosorbent assay; BHS, bighorn sheep; DS, domestic sheep

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catarrhal fever is a lymphoproliferative disease that can show a broad range of symptoms and manifestations. Typically, the disease has an acute outcome and is commonly fatal, but more chronic manifestations have also being recognized (O'Toole and Li, 2014; O'Toole et al., 1997; Russell et al., 2009).

Ovine herpesvirus-2 (OvHV-2) is an MCFV carried by sheep worldwide. It has been generally accepted that both domestic sheep and wild sheep can serve as reservoirs for OvHV-2. This is based on positive results on the OvHV-2 gold-standard diagnostic assay, a PCR that uses primers homologous to the viral tegument gene sequence derived from domestic sheep (Baxter et al., 1993). However, limited published data confirming virus identity in wild sheep is available.

In 2015, an adult female bighorn sheep (*Ovis canadensis*) from Banff National Park, Alberta, Canada was diagnosed with severe dermatitis associated with a MCFV (Slater et al., 2017). The diagnosis of MCF-like skin disease in this animal was based on exclusion of other disease processes, gross and histological lesions, coupled with positive results on the OvHV-2 nested-PCR and the presence of higher levels of OvHV-2 DNA in the skin as compared with asymptomatic bighorn sheep, as tested by OvHV-2 quantitative PCR. Both PCR assays were developed based on OvHV-2 sequences obtained from domestic sheep (Baxter et al., 1993; Hussy et al., 2001). At the time, a partial sequencing (362 base pairs) of the OvHV-2 tegument gene encoded by ORF 75 (GenBank: KX060582) obtained from this animal showed 94.2% identity to the homologous region in OvHV-2 from domestic sheep (GenBank: DQ198083). However, alignments of 19 OvHV-2 ORF 75 sequences, obtained from OvHV-2 worldwide and available in the GenBank database, show 98.6–100% identity in this region. The lower sequence identity in the ORF 75 gene found between the virus carried by the bighorn sheep (94.2%) compared to available OvHV-2 sequences (98.6–100%), supports the idea that the viruses infecting bighorn sheep and domestic sheep might be different. To test this hypothesis, variability in the genome region that allows for taxonomic classification of herpesviruses, the viral DNA polymerase gene between motifs B and C (VanDevanter et al., 1996), was compared across MCFVs and the virus infecting bighorn sheep.

Amplification by PCR of the highly conserved region of the herpesviral DNA polymerase gene was performed on DNA extracted from a respiratory tract mucosa sample collected from the bighorn sheep with MCF-like skin lesions (Slater et al., 2017). The herpesvirus consensus primer PCR was performed as previously described (VanDevanter et al., 1996) and an expected 232 base pairs amplicon was obtained. The amplicon was cloned into pCR4 (Thermo Fisher Scientific) as per the manufacture's recommendation and six clones were sequenced using M13 forward and reverse primers (Eurofins). Identical sequences covering the viral DNA polymerase motifs B to C region were confirmed and the internal 177 base pairs were subsequently compared with OvHV-2 sequences available in GenBank using AlignX (Vector NTI). As illustrated in Fig. 1A, sequence alignments reveled 85.88% nucleotide identity between the MCFV carried by the bighorn sheep (BHS-MCFV) and the one isolated from a domestic sheep nasal secretion (OvHV-2, GenBank: DQ198083). At the protein level, 81.36% amino acid similarity was observed (Fig. 1B). Overall, the partial nucleotide sequence of the DNA polymerase gene from the virus infecting the bighorn sheep shared 84.7–86.4% identity with other 16 sequences of the same region identified in GenBank as of OvHV-2 origin and 65.5–75.1% with other MCFVs (Table S1).

To investigate whether the variation observed was an incidental finding in this particular animal or consistent among bighorn sheep, additional DNA samples from bighorn sheep from flocks in the United States and Canada were evaluated. As described in Table 1, a total of 88 DNA samples were subjected to herpesvirus consensus primer PCR (VanDevanter et al., 1996) and 28 resulted in amplicons of the expected size. Twenty two of these amplicons were cloned and sequenced as described above. Sequencing results showed that 14 amplicons from flocks from Alberta, Canada and Montana, USA shared 100% identity with the nucleotide sequence obtained from the from the first bighorn sheep in the study (BHS-MCFV.1). Five amplicons (MN068216), from animals located in Nevada and California, USA (BHS-MCFV.2), were 100% identical but shared only 98.9% identity (2 out 177 nucleotide mismatches) to the BHS-MCFV.1 sequences (Fig. 1C, Table 1). The remaining three amplicons showed high sequence homology to a type-2 ruminant rhadinovirus, which is a non-MCFV previously reported in bighorn sheep (Li et al., 2005b). To further confirm the presence of a MCFV in the bighorn sheep, serology was performed using serum or plasma samples available from 13 of the animals carrying the BHS-MCFV variants. Serum/plasma samples were tested by a MCF-cELISA, based on the MCFV 15-A epitope, as previously described (Li et al., 2001b). All 13 samples were positive for antibodies to the 15-A, which is expected in animals infected with MCFVs (Li et al., 2001a). The sequencing and serological results are summarized in Table 1.

To confirm the relationships among the MCFVs identified in bighorn sheep and in other hosts, a phylogenetic analysis was performed using representative nucleotide sequences of all known MCFVs, covering 174–177 base pairs of the viral DNA polymerase gene, motifs B to C region. Sequence alignment and phylogenetic analysis were performed online at the T-REX website (Boc et al., 2012). Briefly, sequences were aligned using ClustalW with default settings and RAxML was used to perform Maximum Likelihood Analysis. The resulting tree was imported into FigTreev1.4.4, rooted with SuHV4, a non-MCF Macavirus, and visualized as a radial tree. As expected, the sequences obtained from bighorn sheep grouped together, with OvHV-2 being the closest relative (Fig. 2). Overall, the sequence analysis data is in accordance with the observation that strain-to-strain variations of herpesviruses within singles species is less pronounced than species-to-species variation (McGeoch et al., 2005; VanDevanter et al., 1996). The higher intraspecies similarities observed among viral DNA polymerase gene sequences obtained from either domestic sheep or from bighorn sheep (98–100%) as compared to interspecies similarities (85–86%), provides evidence that the viruses carried by domestic sheep and bighorn sheep are distinct from one another. The occurrence or possibility of interspecies transmission of these MCFVs between bighorn sheep and domestic sheep is currently unknown and requires further experimental investigation.

Evolutionary relationships between members of the MCF subgroup and host species are clearly observed by comparing phylogenetic trees of viral DNA polymerase and host mitochondrial cytochrome b amino acid sequences (Li et al., 2005b). The overall correspondence between the virus and the host branching patterns supports the idea that MCFVs have co-evolved with their respective hosts and their divergence was probably caused by host speciation over long periods of time. In fact, it has been previously demonstrated, and confirmed in this study (Fig. 2), that MCFVs are clustered into two major subgroups that correspond to their reservoir species: the Alcelaphinae/Hippotraginae subgroup and the Caprinae subgroup (Li et al., 2005b). Viruses within each subgroup have certain biological properties in common. For instance, MCFV in the Alcelaphinae/Hippotraginae subgroup can be propagated in cell culture, while the ones in the Caprinae subgroup do not replicate when the same in vitro conditions are applied. Also, transmission of two viruses in the Caprinae subgroup, OvHV-2 and caprine herpesvirus-2, occurs in a similar pattern in sheep and goats, respectively (Li et al., 1998, 2005c). Although there is no information about the epidemiology, virulence, or any other biological properties of the MCFVs found in bighorn sheep, we would expect it to behave similarly to OvHV-2.

Sheep are considered as reservoir hosts of MCFVs and in this sense expected to carry the viruses asymptomatically, which is usually the case. However, with the advancement of molecular diagnostic techniques to identify and quantify these viruses, OvHV-2 has been confirmed to infrequently cause MCF in sheep (Himsworth et al., 2008; Li et al., 2005c; Pesavento et al., 2019; Phillips et al., 2018; Slater et al., 2017) . The bighorn sheep with a MCF-like skin disease described by Slater

Fig. 1. Alignment of partial (motif B to motif C) sequences of the herpesviral DNA polymerase gene obtained from domestic sheep and bighorn sheep. Sequences identified as BHS-MCFV and BHS-MCFV.1 are identical and were obtained from bighorn sheep located in Alberta, Canada (Slater et al., 2017, GenBank: MN068215) and Montana, USA; DS-OvHV-2 was obtained from domestic sheep nasal secretions (Taus et al., 2007, GenBank: DQ198083); and BHS-MCFV.2 from bighorn sheep located in Nevada and California, USA (GenBank: MN068216). A, 85.88% nucleotide identity; B, 81.36% amino acid identity; and C, 98.87% nucleotide identity.

et al. in 2017 was the first animal identified as a carrier of the BHS-MCFV described in this study. Although an association between the MCFV and skin lesions was observed in that animal, the majority of bighorn sheep that carry the virus are expected to be asymptomatic. Better understanding of possible disease outcomes in bighorn sheep and other species following infection with the BHS-MCFV would require experimental challenge studies. However, such studies are difficult to perform due to the necessity of obtaining viral stocks for inoculum directly from bighorn sheep during shedding episodes.

It is important to note that because viral DNA can be amplified from both domestic and wild sheep using PCR assays designed for OvHV-2 (Baxter et al., 1993; Himsworth et al., 2008; Slater et al., 2017; Traul et al., 2007), it has been always assumed that both domestic sheep and wild sheep serve as reservoirs for OvHV-2. Contrary to this assumption, our results show that bighorn sheep carry a MCFV that is distinct from OvHV-2. To evaluate the sequence region where the primers of the gold standard OvHV-2 nested PCR bind, partial sequence of the tegument gene from BHS-MCFV was evaluated. Briefly, the sequence of interest was amplified by PCR using the following forward, Ov-2 ORF75 +52 F: 5′ CAGGATTACAGACAGACATTC 3′, and reverse primers, Ov-2 ORF75 1805 R: 5′ TGTCCTGAATGAGTGTGTGC 3′, and the obtained amplicon was sequenced (GenBank: MN068217). Nucleotide mismatches compared to the OvHV-2 sequence (GenBank: DQ198083) were observed only within the binding region of the reverse primer, 556, used in the OvHV-2 nested PCR (Fig. 3). In an attempt to evaluate the sensitivity of the OvHV-2 nested PCR for BHS-MCFV, known copies of plasmid DNA containing the amplicon region for either OvHV-2 or BHS-MCFV were used as templates and the assay performed as previously described (Li et al., 1995). No difference in the PCR performance was noted with either plasmid, even when as low as 10 copies of plasmid DNA was used per reaction. It is important to note that the PCR may perform differently with plasmid DNA templates and DNA isolated from animal tissues, which usually carry higher amounts of PCR inhibitors. Therefore, it is possible that in samples from animals the primer mismatched has a more pronounced effect, resulting in decreased PCR sensitivity and increased number of false negative results. Although the results suggest that the OvHV-2 nested PCR may still be used as a gold standard to detect MCF viruses in sheep, negative results need to be interpreted

Table 1
Bighorn sheep samples from multiple locations in the US and Canada tested for malignant catarrhal fever viruses (MCFV) using DNA analysis and serology. DNA analysis was based on sequence of a portion of the viral DNA polymerase gene and serology was based on antibodies to the viral 15-A epitope.

Location	DNA					Serum/plasma	
	Herpesvirus Consensus Primer PCR[a]		Sequencing			MCF-cELISA[b]	
	# of samples tested	# of samples with expected amplification	# of samples with homology to a MCFV / # of samples sequenced	% identity to BHS-MCFV.1 (GenBank: MN068215)	% identity to OvHV-2 from DS (GenBank: DQ198083)	# of samples tested	Samples positive to the 15-A epitope
Alberta, CA[c]	35	12	8/9	100.00	85.88	6	6
Montana, US	43	9	6/6	100.00	85.88	6	6
California, US	1	1	1/1	98.87	85.88	1	1
Nevada, US	9	6	4/6	98.87	85.88	n/a	n/a
Total	88	28	19/22			13	13

BHS, bighorn sheep.
MCFV, malignant catarrhal fever virus.
DS, domestic sheep.
n/a, not available.
[a] VanDevanter et al., 1996.
[b] Li, et al., 1994.
[c] Banff National Park, AB, Canada.

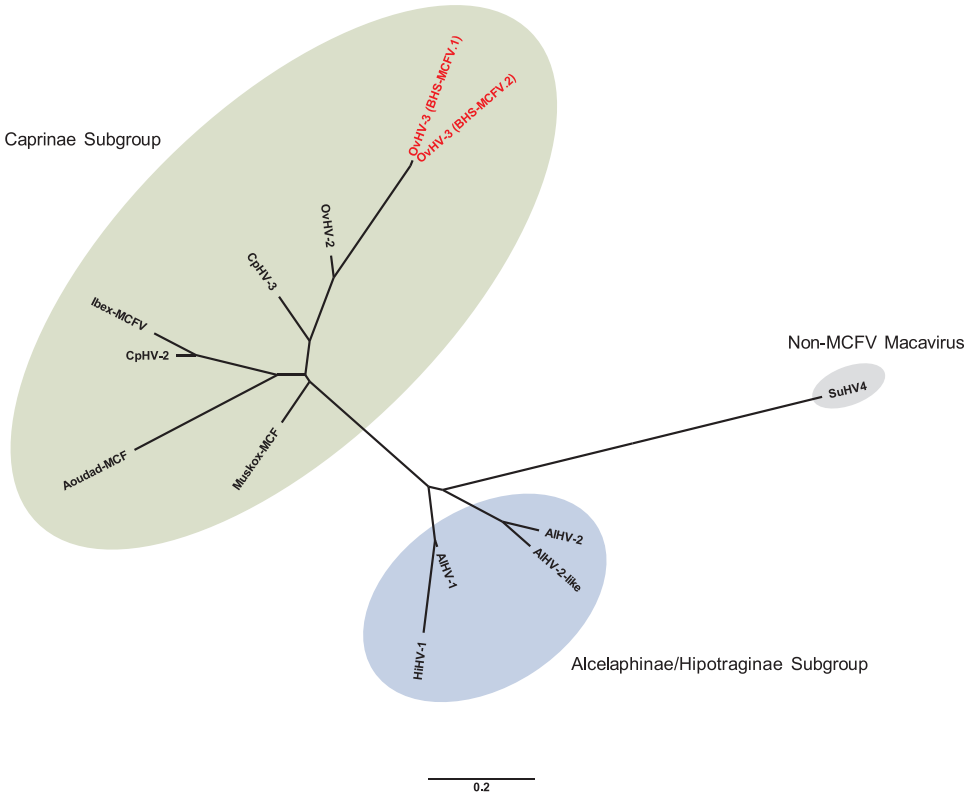

Fig. 2. Phylogenetic relationship of malignant catarrhal fever viruses (MCFV). Radial tree rooted with SuHV4, a non-MCF macavirus, was generated in FigTreev1.4.4 following alignment and Maximum Likelihood Analysis of nucleotide sequences representing all recognized MCFV. The viruses recognized in this study are indicated in red. Highlighted subgroups refer to the host species that serve as reservoirs for each cluster of viruses.

with caution since the detection of BHS-MCFV in field samples may be compromised. Our laboratory is currently working on modifications in the assay by either incorporating degenerated nucleotides in the primers or using new primers specific to each virus aiming to obtain more sensitive and specific ways to detect and differentiate MCFVs in sheep.

In summary, here we described the recognition of a new gammaherpesvirus in bighorn sheep. The virus follows genetic and antigenic criteria to be classified as a member of the MCF subgroup of Macaviruses: 85.88% sequence homology to a conserved region of the viral DNA polymerase gene of OvHV-2, the closest known relative, and presence of antibodies to the viral 15-A epitope in infected animals. So far, there is no evidence that cross-species infection or transmission occurs between the MCF viruses carried by domestic sheep and bighorn sheep. Detection and further analysis of infected bighorn sheep in diverse populations will help clarify circulation patterns of this virus, and determine the risk for MCF-susceptible species. Considering the relationship to OvHV-2 and the fact that the newly recognized virus is carried by a distinct natural host within the genus *Ovis*, we suggest referring to the MCF virus carried by bighorn sheep as ovine herpesvirus-3 (OvHV-3).

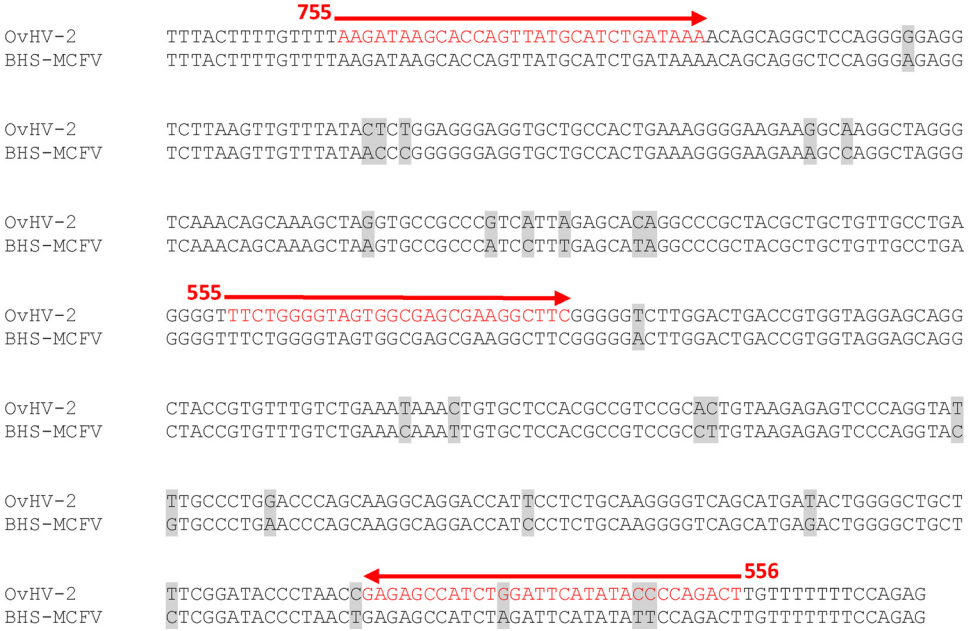

Fig. 3. Alignment of partial sequences of the tegument gene of DS-OvHV-2 and BHS-MCFV indicating the binding sites from primers 775, 555, and 556 used in the OvHV-2 nested PCR (Baxter et al., 1993), which is considered the gold-standard for diagnosis of sheep-associated MCF. Primer sequences are indicated in red and primer binding sites and orientation indicated by arrows.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197729.

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