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HERPESVIRUSES INCLUDING NOVEL GAMMAHERPESVIRUSES ARE WIDESPREAD AMONG PHOCID SEAL SPECIES IN CANADA

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ABSTRACT: Little is known about herpesviruses in Canadian pinnipeds. We measured prevalence of antibodies to herpesviruses in the sera from Canadian phocid seals by an indirect enzyme-linked immunosorbent assay. Wild harbor seals (*Phoca vitulina*) and captive harbor seals were positive for antibodies to *Phocid herpesvirus 1* (PhoHV-1) at prevalences of 91% and 100%, respectively. Sera from wild hooded seals (*Cystophora cristata*), harp seals (*Pagophilus groenlandica*), and grey seals (*Halichoerus grypus*) were positive for antibodies to PhoHV-1 antigenically related herpesvirus antigens at 73%, 79%, and 96%, respectively. We isolated new herpesviruses in cell culture from two hunter-harvested ringed seals (*Pusa hispida*) in poor body condition from Ulukhaktok, Northwest Territories, Canada; one lethargic hooded seal from the St. Lawrence Estuary, Québec, Canada; and one captive, asymptomatic harp seal from the Magdalen Islands, Québec. Partial sequencing of the herpesvirus DNA polymerase gene revealed that all four virus isolates were closely related to PhoHV-2, a member of the *Gammaherpesvirinae* subfamily, with nucleotide similarity ranging between 92.8% and 95.3%. The new seal herpesviruses were genetically related to other known pinniped herpesviruses, such as PhoHV-1, *Otariid herpesvirus 3*, Hawaiian monk (*Monachus schauinslandi*) seal herpesvirus, and *Phocid herpesvirus 5* with 47–48%, 55%, 77%, and 70–77% nucleotide similarities, respectively. The harp seal herpesvirus and both ringed seal herpesviruses were almost identical to each other, whereas the hooded seal herpesvirus was genetically different from the three others (92.8% nucleotide similarity), indicating detection of at least two novel seal herpesviruses. These findings are the first isolation, partial genome sequencing, and identification of seal gammaherpesviruses in three species of Canadian phocid seals; two species of which were suspected of exposure to one or more antigenically related herpesviruses based on serologic analyses.

Key words: DPOL gene, ELISA, herpesvirus, phocid seals, serologic survey, virus isolation.

INTRODUCTION

Six phocid seal species are found in Canadian waters, including harp (*Pagophilus groenlandica*), hooded (*Cystophora cristata*), ringed (*Pusa hispida*), harbor (*Phoca vitulina*), bearded (*Erignathus barbatus*), and grey (*Halichoerus grypus*) seals. Canadian phocids were reported to be infected with several viruses, including influenza A virus (Nielsen et al. 2001), a picornavirus (Kapoor et al. 2008), and a paramyxovirus (Daoust et al. 1993). Serologic data also suggest that Canadian harbor seals were exposed to one

or more herpesviruses (Goldstein et al. 2003; Himworth et al. 2010).

Herpesviruses have a linear double-stranded DNA genome of approximately 130–230 kilobases (kb) and are classified in the *Herpesviridae* family within three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Seal herpesvirus infection was first detected in 1984 in harbor seal (*Phoca vitulina*) pups being treated in a rehabilitation center (Osterhaus et al. 1985). Subsequently, other alpha- and gammaherpesviruses were detected in European and North American

TABLE 1. Prevalence of antibodies cross-reactive to phocid herpesvirus 1 by enzyme-linked immunosorbent assay in eastern Canadian phocid seals (wild and captive) by age and sex.

Species	Age ^a and sex	No. positive/No. tested (prevalence [%])
Harbor seal (<i>Phoca vitulina</i>)	Adult male (5 yr)	1/1 (100)
	Adult female (4 yr)	1/1 (100)
	1 Juvenile male (2 yr); (1 unknown age)	2/2 (100)
	YOY male (3–5 mo)	4/4 (100)
	YOY female (4–5 mo)	2/3 (67)
Total		10/11 (91)
Harbor seal (captive; <i>Phoca vitulina</i>)	Adult male (7–13 yr)	3/3 (100)
	Adult female (12–27 yr)	4/4 (100)
	YOY female (7 mo)	1/1 (100)
Total		8/8 (100)
Hooded seal (<i>Cystophora cristata</i>)	Adult male (9–23 yr)	11/15 (73)
	Adult female (7–22 yr)	11/15 (73)
Total		22/30 (73)
Harp seal (<i>Pagophilus groenlandica</i>)	Adult male (6–22 yr)	13/14 (93)
	Adult female (4–8 yr)	10/15 (67)
Total		23/29 (79)
Grey seal (<i>Halichoerus grypus</i>)	Adult male (8–25 yr)	15/15 (100)
	Adult female (5–27 yr)	16/16 (100)
	Juvenile male (1–5 yr)	6/6 (100)
	Juvenile female (3 yr)	1/1 (100)
	YOY male (1–9 mo)	5/5 (100)
YOY female (9 mo)	0/2 (0)	
Total		43/45 (96)

^a Age (years or months) based on growth layer groups in sectioned canine or incisor teeth. One grey, two harp, and one harbor seal were determined to be juveniles or adults based on standard length. The estimated age in months of young-of-the-year (YOY) was based on month of birth (May for harbor seals, January for grey seals).

pinnipeds (Harder et al. 1996; King et al. 1998; Martina et al. 2003). *Otarine herpesvirus 1* is thought to be associated with urogenital carcinoma in California sea lions (*Zalophus californianus*; Buckles et al. 2007) and in a captive South American fur seal (*Arctocephalus australis*; Dagleish et al. 2013), but other cofactors are thought to be involved in cancer development. *Phocid herpesvirus 1* can cause serious illness in harbor seals, particularly in rehabilitation centers, where young pups are under stress or may have other health problems leading to susceptibility to disease and death (Gulland et al. 1997). Gammaherpesviruses were detected in samples from healthy and sick pinnipeds of all ages (Martina et al. 2003). *Phocid herpesvirus 1* can be transmitted between harbor seals within rehabilitation centers and on rookeries (Martina et al. 2002; Goldstein et al. 2004;

Himworth et al. 2010). We present serologic data demonstrating that four species of Canadian phocids were exposed to one or more antigenically related herpesviruses and present the detection and partial characterization of at least two novel gammaherpesviruses in three Canadian phocid species.

MATERIALS AND METHODS

As part of serologic and health assessment surveys, seals were shot ($n=11$ harbor seals, 21 grey seals, 29 harp seals) or live-captured, bled, and released ($n=30$ hooded seals, 24 grey seals), with a scientific permit issued by Fisheries and Oceans Canada (DFO), from the Gulf of St. Lawrence and Estuary (Table 1). Harp seals were sampled in March 1996–97 and March 1999–2001 and hooded seals in March 1996–97 near the Magdalen Islands (47°82'39"N, 61°85'29"W). Harbor seals were sampled at Bic, Quebec (QC; 48°82'39"N,

68°55'39"W), in May and August 1995; at Murray Harbour, Prince Edward Island (PEI; 46°0'24"N, 62°31'32"W), and Governors Island, PEI (46°8'9"N, 63°3'32"W), in September 2000; and near Charlottetown, PEI (46°14'24"N, 63°8'23"W), in October 2001. Grey seals were sampled on Hay Island, Nova Scotia (NS; 46°80'19"N, 59°84'19"W), and near Port Hood, NS (46°80'19"N, 61°83'29"W), in January and February 1996; on Amet Island, New Brunswick (NB; 45°50'N, 63°10'W), and Governors Island in September 2000; on Portage Island, Miramichi Bay, NB (47°7'N, 65°10'W), in June 2000; and near Charlottetown in June, September, and October 2001. Harbor seals ($n=8$) held permanently at two aquaria, Aquarium du Québec, Ste-Foy (46°45'3"N, 71°17'18"W), and New Brunswick Aquarium and Marine Centre, Shippagan, NB (47°44'38"N, 64°43'4"W), were also sampled in November and December 1996 (Table 1).

As part of a surveillance program for viruses in marine mammals in Canada, virus isolation attempts were performed on samples from several phocid seals (lungs and lymph nodes from hunter-harvested animals and blood from live-captured animals), including four herpesvirus-positive cases: lung and lymph nodes from two ringed seals (out of 16 tested) and blood samples collected from one hooded seal and one harp seal. The two ringed seals (in poor body condition) were hunter-harvested during a long-term study with subsistence hunters in the western Canadian Arctic (Harwood et al. 2012). The adult female hooded seal was reported on shore at Pointe-au-Père, QC (48°31'01"N, 68°28'04"W), on 21 November 2004, and appeared in good body condition but was lethargic and easily approached and net-captured for blood collection then released. The weaned female harp seal pup was captured on the ice near the Magdalen Islands in March 2004 and was held in captivity at DFO, Mont-Joli, for a lungworm experiment (see Piché et al. 2010 for details on husbandry). This healthy harp seal was a control animal and a blood sample was obtained on 16 August 2004. Tissue samples from the two ringed seals were held at -20 C for 2 mo before being shipped to DFO, Winnipeg, for virus isolation. Blood from the hooded and harp seals were collected in heparin-treated Vacutainers® (Becton, Dickinson, and Company, Franklin Lakes, New Jersey, USA) and shipped on ice to DFO, Winnipeg for virus isolation.

For the serologic survey, blood was obtained from a jugular vein, the heart, or a lumbar intravertebral extradural vein using 50–152 mm, 18-gauge needles and syringes and, for serologic assays, transferred to untreated Vacutainers.

Blood samples were kept warm until clotted and centrifuged within 6 h of collection for 20 min at $3,000 \times G$. Sera were removed to sterile 1- or 2-mL cryogenic vials and stored at -80 C until assayed for herpesvirus antibodies by indirect enzyme-linked immunosorbent assay (ELISA). The antigen used in the ELISA assay, PhoHV-1 Pacific isolate HS950, was propagated and purified as described by Goldstein et al. (2003). Microtiter plates (Probind®, Falcon, Becton Dickinson) were coated overnight at 4 C with 1.4 µg/mL of purified PhoHV-1 antigen. The plates were blocked with 1% bovine serum albumin (Sigma, St. Louis, Missouri, USA) for 1 h at room temperature, followed by incubation of the serum samples for 1 h at room temperature, and antibody binding was detected by sequential incubation with 0.5 µg/mL biotinylated anti-grey seal (immunoglobulin G [IgG]-specific monoclonal antibody-H49a that cross-reacts with harbor seal IgG; King et al. 1993) for 1 h at room temperature, 0.83 µg/mL horseradish peroxidase-conjugated streptavidin (HRP-streptavidin, Zymed, San Francisco, California, USA) for 20 min at room temperature, and *O*-phenylenediamine dihydrochloride (Sigma) for 25 min at room temperature, in the dark for development. Optical densities of the color-change proportional to the concentration of PhoHV-1-specific antibodies in the samples were read at 490 nm with an ultraviolet max kinetic microplate reader. Samples were tested in duplicate at 1:100 dilution and compared with a positive reference sample with a PhoHV-1-specific antibody level designated at 100 units/mL. Results were recorded as a percentage of this standard, and samples with antibody concentrations >5 units/mL were considered positive to PhoHV-1, <1 unit/mL negative, and $\geq 1 \leq 5$ units/mL equivocal for harbor seal anti-PhoHV-1 antibodies (Goldstein et al. 2003). As this assay used whole PhoHV-1 antigens not validated for harp, hooded, and grey seals and because the ELISA cannot distinguish between PhoHV-1-specific antibodies and cross-reacting antibodies to other antigenically related herpesviruses, we conservatively considered values >10.0 units/mL positive for unidentified herpesvirus, values <1 units/mL and equivocal values ($\geq 1 \leq 10$ units/mL) negative in these three species (Table 1). Antibody binding, using the biotinylated anti-grey seal IgG-specific monoclonal antibody H49a, was confirmed, ensuring cross-reaction with harp, hooded, and ringed seals IgG.

Seal ages were determined by counting the number of growth layer groups (GLGs) in the dentine or cementum of lower canine or incisor teeth sectioned longitudinally (where one GLG=1 yr old; Smith 1973; Measures et al.

TABLE 2. Herpesvirus strains isolated from three phocid seal species in Canada, 2000–04. Only positive herpesvirus nested PCR results are shown.

Case no. (GenBank accession)	Virus name	Species	Location (latitude and longitude)	Date of collection	Sex	Age	Health status	Tissue	Isolation method
FMV04-1493871 (KF466474)	Hooded seal herpesvirus (HoSHV)	Hooded seal (<i>Cystophora cristata</i>)	Pointe-au-Père, Quebec (48°31'01"N, 68°28'04"W)	November 2004	Female	Adult	Lethargic	Peripheral blood mononuclear cells	Cocultivated with Vero cells
FMV04-1493874 (KF466473)	Harp seal herpesvirus (HaSHV)	Harp seal (<i>Pagophilus groenlandica</i>)	Mont Joli, via Magdalen Islands, Quebec (47°52'39"N, 61°55'29"W)	August 2004	Female	5 mo	Healthy	Peripheral blood mononuclear cells	Cocultivated with Vero cells
FMV00-1493869 (KF466472)	Ringed seal herpesvirus (RiSHV)	Ringed seal (<i>Pusa hispida</i>)	Uluhaktok, NT ^a (70°44'11"N, 117°46'05"W)	July 2000	Male	2 yr	Emaciated, thin fat, LMD ^b extremely low	Lung	Homogenized tissue inoculated onto PSK ^d
FMV02-1493868 (KF466471)	Ringed seal herpesvirus (RiSHV)	Ringed seal (<i>Pusa hispida</i>)	Uluhaktok, NT ^a (70°44'11"N, 117°46'05"W)	July 2002	Male	5 mo	Reduced LMD ^c compared with other ringed seals	Lymph node	Homogenized tissue inoculated onto PSK ^d

^a NT = Northwest Territories.

^b LMD = length-mass-fat depth index of this seal = 6.78; LMD of three seals of same age and date of collection: 20.73 ± 0.92 (mean ± SD).

^c LMD = length-mass-fat depth index of this seal = 13.3; LMD of 11 seals of same age and date of collection: 14.3 ± 4.2 (mean ± SD).

^d PSK = primary harp seal kidney cells.

2004). Young-of-the-year (YOY) seals or pups were in their first year of life (age <1 yr); juveniles were age ≥ 1 yr and sexually immature; adults were sexually mature and aged 4–8 yr for male or 4–6 yr for female harp seals; 4–6 yr for male and 3–4 yr for female hooded seals; 6 yr for male and 4 yr for female grey seals; and 6 yr for male and 3–4 yr for female harbor seals (Measures et al. 2004). One grey, two harp, and one harbor seals were determined to be juveniles or adults based on standard nose-to-tail length. The estimated age in months of YOYs was based on peak month of birth (May for harbor, January for grey seals; see earlier references; Table 1).

For ringed seals, the standard nose-to-tail length (+1.25 cm), axillary girth, and hip girth were measured (American Society of Mammalogists 1967). Body weight to the nearest 0.5 kg was measured using a spring-dial scale suspended from a tripod. Fat thickness (+0.5 cm) was measured midventral at the sternum and at the hip (60% of distance from nose to tail). Body condition was calculated using a length-mass-fat depth (LMD) index (Ryg et al. 1990):

$$\text{LMD}(\text{m}^{1.5}/\text{kg}^{0.5}\text{per year}) = \sqrt{(L/M)} \times (d \times 100) \quad (1)$$

where L = standard length (m), M = body weight (kg), and d = fat thickness (m) at the hip.

Virus isolations were attempted with seal samples collected over many years, using Vero cells and primary harp seal kidney cells, as previously described (Nielsen et al. 2008; Tuomi et al. 2014). Following inoculation and incubation, some cells became swollen and rounded, compared with controls, 10–14 d postinfection in both cell lines. This cytopathic effect (CPE) continued for another week, until all cells became detached. This progressive CPE could be transmitted to fresh cell cultures inoculated with CPE-positive cell lysates. Cell lysates were tested with a pan-herpesvirus-nested PCR assay (nPCR), as described by VanDevanter et al. (1996), to establish whether herpesviruses were isolated. The nPCR assay targets the herpesvirus DNA polymerase (DPOL) gene and is able to detect a broad range of herpesvirus species. Four seal cases (one live-captured hooded seal, one captive harp seal, and two hunter-harvested ringed seals) were nPCR positive, confirming the isolation of herpesviruses (Table 2). Only the pan-herpesvirus PCR-positive virus isolates are listed in Table 2.

One isolated herpesvirus strain was further sequenced using high-throughput sequencing. The HaSHV strain was concentrated by ultracentrifugation (Bellehumeur et al. 2013),

recovered in TNE buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, and 0.1 mM EDTA), and treated with propidium monoazide to lower genomic DNA contamination (100 μM ; 5 min at room temperature and 5 min on ice, followed by 10 min exposition to two 500-W halogen lights sources, 20 cm from the light source), as previously described (Bellehumeur et al. 2015a). Total genetic material was extracted with two volumes of phenol-chloroform (UltraPure™ buffer-saturated phenol [pH 7.6–7.8] solution [Invitrogen, Burlington, Ontario, Canada], mixed with chloroform and isoamyl alcohol), cleaned with one volume of chloroform, and precipitated with two volumes of ethanol and 1/10 volume of sodium acetate (3 M, pH 5.2). Recovered DNA/RNA was resuspended in 50 μL of RNase-free water.

The DNA/RNA sample was amplified with a random PCR (Chen et al. 2011) and end-repaired and A-tailed using KAPA high-throughput library preparation kit with SPRI solution and standard PCR library amplification/Illumina series (Kapa Biosystems, Wilmington, Massachusetts, USA). Illumina TruSeq HT dual-indexed adapters (Illumina, San Diego, California, USA) were ligated to the random PCR amplicons, and the library was amplified with the Kapa kit. After the final cleanup, the quality of the library was assessed on high-sensitivity DNA chips using a 2100 BioAnalyzer (Agilent, Santa Clara, California, USA). The library was sequenced with an Illumina MiSeq (2 \times 300 paired-end reads, dual-indexed). Raw-sequencing readings were assembled with the A5-miseq bacterial genome assembly pipeline (Cornell-University-Library 2014).

RESULTS

The ELISA indicated that four species of phocid seals from the east coast of Canada commonly have cross-reactive antibodies against PhoHV-1, suggesting that animals have been exposed to at least one PhoHV-1 antigenically related herpesvirus. Of 123 phocid seals tested, 106 (86%) were antibody positive. The antibody prevalence was high (73% to 96%) with an overall prevalence of 91% in wild phocids and 100% in captive harbor seals (Table 1). Sera from hooded, harp, and grey seals tested positive for anti-PhoHV-1 antibodies using the ELISA with a conservative positive cutoff. However, the specific

herpesvirus or herpesviruses to which each animal was exposed cannot be identified based on this serology. Because the ELISA used in this study was developed for PhoHV-1 and validated in harbor seals, wild and captive harbor seals had antibodies to PhoHV-1 with three wild seals (one adult and two juveniles) and one captive adult having antibody concentrations >100 units/mL. Antibodies were detected in almost all age-sex classes tested. This included newborn seals as young as 1–9 mo and one harbor seal born in captivity, as well as in other seals as old as 27 yr (Table 1). There were significant differences in median antibody concentrations between sexes within adult hooded and grey seals (females > males) and harp seals (males > females; Mann-Whitney rank sum test, $P < 0.001$) with hooded and grey seals having up to 580 units/mL compared with harp seals with values <50 units/mL.

Both hooded seal and harp seal herpesviruses were isolated from peripheral blood mononuclear cells of heparinized blood samples (Table 2). The nPCR-positive adult hooded seal found alive on shore appeared in good condition although lethargic (Table 2). The nPCR-positive harp seal pup held in a seal-holding facility was in good health while in captivity (Table 2). Both ringed seal herpesviruses were isolated from lung and lymph node tissue homogenates inoculated into cell cultures in spite of those tissues being stored at -20 C for an extended time. The LMD index of the two positive ringed seals was compared with the mean LMD of 14 ringed seals that were negative for herpesvirus. These 14 negative ringed seals were of the same age-class and sampled in the same month and year as the positive ringed seals to which they were compared. One pup was positive with an LMD (6.78), which was considerably less than the mean LMD of three pups that were negative (mean LMD 20.73). The hunter noted that the positive pup had an empty stomach and described it as a “starveling.” The other positive ringed seal was a subadult with

a LMD (13.3), which was slightly lower than the mean LMD of 11 subadult ringed seals that were negative (mean LMD 14.3). There was no evidence of lungworm infection or other harvest-recorded abnormalities in the positive subadult (Table 2).

The nPCR products were sequenced. The sequences were submitted to GenBank Basic Local Alignment Search Tool (BLAST) for comparison (Altschul et al. 1997) and revealed the presence of gammaherpesviruses (data not shown). To obtain a longer nucleotide (nt) sequence length of the herpesvirus DPOL gene, DNA recovered from the first step of the nPCR reaction was sequenced. The DNA viral genome sequences (varying from 636 to 685 base pairs [bp]) were deposited into the GenBank database (accessions KF466471–KF466474; Table 2).

Both ringed seal herpesvirus nt sequences were 100% identical. One nt difference was observed between the harp seal herpesvirus [HaSHV] and the ringed seal herpesvirus [RiSHV] isolates (data not shown). However, the hooded seal herpesvirus (HoSHV) had significant nt sequence differences when compared with the three other sequences (92.8% nt similarity). Deduced amino acid (aa) sequences were 97.8% similar between HoSHV and the three other seal herpesviruses. These nt and aa sequence analyses revealed at least two novel seal herpesviruses. The closest nt similarity for HoSHV, RiSHV, and HaSHV were with the DPOL gene of the *Phocid herpesvirus 2* (PhoHV-2; from 92.8% to 95.3%, accession GQ429152; data not shown). Other seal herpesviruses previously documented were related to the seal herpesviruses reported in this manuscript, including the *Otariid herpesvirus 1* and 3 (78–82% and 55% nt identity; AF236050 and JX080682, respectively), the PhoHV-5 (70–77% nt identity; GQ429153) and the Hawaiian monk seal PhoHV-3 (77% nt identity; DQ093191).

Phylogenetic trees containing 45 to 51 herpesvirus DPOL partial nt sequences (with 714 or 396 nt DNA consensus

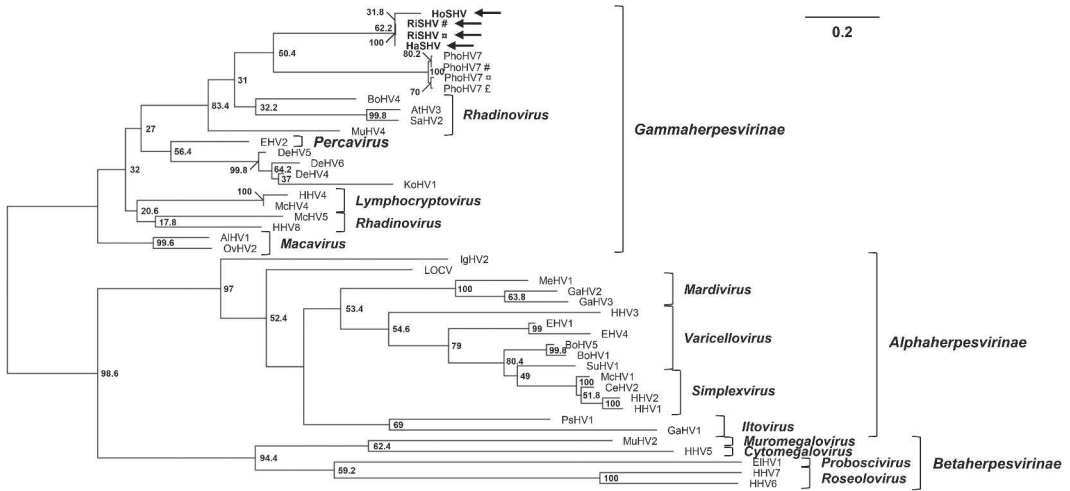


FIGURE 1. Phylogenetic tree of herpesviral partial 714 nucleotides DNA polymerase (DPOL) genes consensus sequences. A phylogenetic tree containing 45 herpesvirus DPOL partial nucleotide (nt) sequences (with a 714-nt DNA consensus sequence) was generated by the PHYLM (PHYlogenetic inferences using maximum likelihood) method with 500 bootstrap replicates with Geneious Pro (version 5.6.6) software using a MAFFT alignment. Bootstrap confidence levels are indicated at the nodes. The scale bar reflects evolutionary distance, measured in units of substitution per nucleotide site. Herpesvirus subfamilies and herpesvirus genera are identified by brackets. The arrows indicate the novel phocid seal herpesviruses. Sequences from GenBank were selected, as previously described (Bellehumeur et al. 2015b), and included *Alcelaphine herpesvirus* (HV) 1 (AlHV-1; NC_002531), *Ateline HV3* (AtHV-3; NC_001987), *Bovine HV1* (BoHV-1; NC_001847), *BoHV-4* (NC_002665), *BoHV-5* (NC_005261), *Cercopithecine HV2* (CeHV-2; AY714813), *Delphinid HV4* (DeHV-4; AY952777), *DeHV-5* (AY952776), *DeHV-6* (DQ288666), *Elephantid HV1* (ElHV-1; AF322977), *Equid HV1* (EHV-1; NC_001491), *EHV-2* (NC_001650), *EHV-4* (NC_001844), *Gallid HV1* (GaHV-1; NC_006623), *GaHV-2* (NC_002229), *GaHV-3* (NC_002577), *Harp seal HV* (HaSHV; KF466473), *Human HV1* (HHV-1; NC_001806), *HHV-2* (NC_001798), *HHV-3* (NC_001348), *HHV-4* (NC_007605), *HHV-5* (NC_006273), *HHV-6* (NC_001664), *HHV-7* (NC_001716), *HHV-8* (NC_00933), *Hooded seal HV* (HoSHV; KF466474), *Iguanid HV2* (IgHV-2; AY236869), *Kogiid HV1* (KoHV-1; AY949830), *Loggerhead orocutaneous HV* (LOCV; EU004542), *Macacine HV1* (McHV-1; NC_004812), *McHV-4* (NC_006146), *McHV-5* (NC_003401), *Meleagrid HV1* (MeHV-1; NC_002641), *Murid HV2* (MuHV-2; NC_002512), *MuHV-4* (NC_001826), *Ovine HV2* (OvHV-2; NC_007646), *Phocid HV7* (PhoHV-7; KP163991), *PhoHV-7[#]* (KM262784), *PhoHV-7[□]* (KP163999), *PhoHV-7[△]* (KP163996), *Psittacid HV1* (PsHV-1; NC_005264), *Ringed seal HV* (RiSHV[#]; KF466471 and RiSHV[□]; KF466472), *Saimiriine HV2* (SaHV2; NC_001350), and *Suid HV1* (SuHV-1; NC_006151).

sequences) were generated by the PHYLM (PHYlogenetic inferences using maximum likelihood) method (Guindon and Gascuel 2003) with 500 bootstrap replicates with Geneious Pro (version 5.6.6; Biomatters, Auckland, New Zealand; Biomatters 2014) software using MAFFT alignment (Katoh et al. 2002; Figs. 1, 2). The phylogenetic tree with the longer nt consensus sequence (714 nt; Fig. 1) and the BLAST results indicated that all the newly identified seal herpesviruses were closely related to the *Rhadinovirus* genus of the *Gammaherpesvirinae* subfamily with a bootstrap value of 83.4 between the *Murid herpesvirus 4* (MuHV-4; a rhadinovirus) and several

other viruses, including three other rhadinoviruses (Fig. 1). To be able to compare the RiSHV, HaSHV, and HoSHV with other reported phocid herpesviruses, a shorter consensus sequence of 396 nt was analyzed. We found RiSHV, HaSHV, and HoSHV to be closely related to other phocid herpesviruses, such as PhoHV-2, -3, -5, and -7 (Fig. 2). However, the nt similarity of RiSHV, HaSHV, and HoSHV compared with PhoHV-1, an alphaherpesvirus, was low (47–48%).

Two large nt sequences (108,857 and 8,589 nt) were obtained from the isolated HaSHV and submitted to GenBank (accession KP136799). Both sequences have

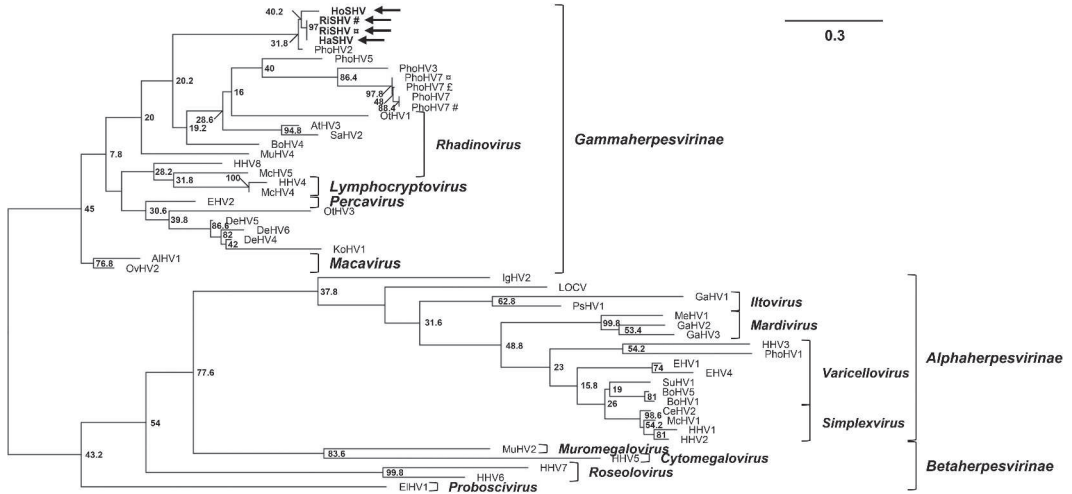


FIGURE 2. Phylogenetic tree of herpesviral partial 396 nucleotides DNA polymerase (DPOL) genes consensus sequences. A phylogenetic tree containing 51 herpesvirus DPOL partial nucleotide (nt) sequences (with a 396 nt DNA consensus sequence) was generated by the PHYLM method with 500 bootstrap replicates with Geneious Pro (version 5.6.6) software using a MAFFT alignment. Bootstrap confidence levels are indicated at the nodes. The scale bar reflects evolutionary distance, measured in units of substitution per nt site. Herpesvirus subfamilies and herpesvirus genera are identified by brackets. The arrows indicate the novel phocid seal herpesviruses. Sequences from GenBank were selected as previously described (Bellehumeur et al. 2015b) and include *Alcelaphine herpesvirus* (HV) 1 (AIHV-1; NC_002531), *Ateline HV3* (AtHV-3; NC_001987), *Bovine HV1* (BoHV-1; NC_001847), *BoHV-4* (NC_002665), *BoHV-5* (NC_005261), *Cercopithecine HV2* (CeHV-2; AY714813), *Delphinid HV4* (DeHV-4; AY952777), *DeHV-5* (AY952776), *DeHV-6* (DQ288666), *Elephantid HV1* (ElHV-1; AF322977), *Equid HV1* (EHV-1; NC_001491), *EHV-2* (NC_001650), *EHV-4* (NC_001844), *Gallid HV1* (GaHV-1; NC_006623), *GaHV-2* (NC_002229), *GaHV-3* (NC_002577), *Harp seal HV* (HaSHV; KF466473), *Human HV1* (HHV-1; NC_001806), *HHV-2* (NC_001798), *HHV-3* (NC_001348), *HHV-4* (NC_007605), *HHV-5* (NC_006273), *HHV-6* (NC_001664), *HHV-7* (NC_001716), *HHV-8* (NC_00933), *Hooded seal HV* (HoSHV; KF466474), *Iguanid HV2* (IgHV-2; AY236869), *Kogiid HV1* (KoHV-1; AY949830), *Loggerhead orocutaneous HV* (LOCV; EU004542), *Macacine HV1* (McHV-1; NC_004812), *McHV-4* (NC_006146), *McHV-5* (NC_003401), *Meleagrid HV1* (MeHV-1; NC_002641), *Murid HV2* (MuHV-2; NC_002512), *MuHV-4* (NC_001826), *Otariid HV1* (OtHV-1; AF236050), *OtHV-3* (JX080682), *Ovine HV2* (OvHV-2; NC_007646), *Phocid HV1* (PhoHV-1; PHU92269), *PhoHV-2* (GQ429152), *PhoHV-3* (DQ093191), *PhoHV-5* (GQ429153), *PhoHV-7* (KP163991), *PhoHV-7[#]* (KM262784), *PhoHV-7^o* (KP163999), *PhoHV-7^e* (KP163996), *Psittacid HV1* (PsHV-1; NC_005264), *Ringed seal HV* (RiSHV[#]; KF466471 and RiSHV^o; KF466472), *Saimirine HV2* (SaHV-2; NC_001350), and *Suid HV1* (SuHV-1; NC_006151).

a combined G:C content of 40% (39.7 and 43.9%, respectively). Multiple open reading frames from the larger sequence were identified and were homologous to herpesvirus genes (Fig. 3). The full viral genome sequencing of the HoSHV strain was attempted but was unsuccessful because HTS was unable to amplify the entire genome.

DISCUSSION

Because the ELISA we used was developed for PhoHV-1 and validated in harbor

seals, it is reasonable to assume that our serologic harbor seal results identified antibodies reactive to PhoHV-1. Our harbor seal serologic results were similar to those of Goldstein et al. (2003), except we observed no apparent increase with age (by months or years of age) for harbor seals. However, our ELISA assay was not validated in grey, harp, and hooded seals. Therefore, an ELISA-positive result may indicate the presence of cross-reactive antibodies to one or more PhoHV-1 antigenically related herpesviruses in grey, harp, and hooded seals. Consequently, the specific herpesvirus to which animals were

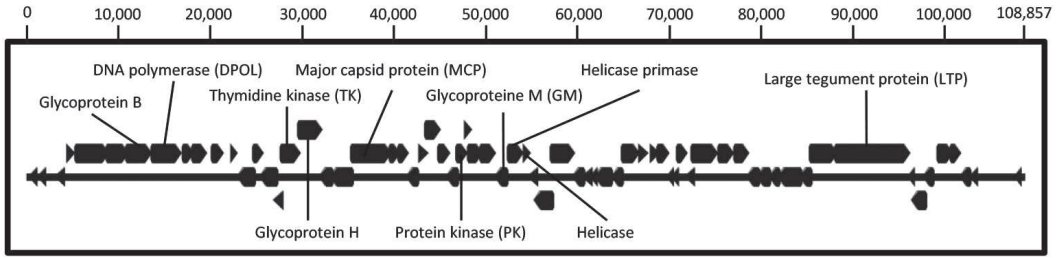


FIGURE 3. Schematic representation of the *Harp seal herpesvirus* sequence recovered by high-throughput sequencing. The longest harp seal DNA fragment (108,857 nucleotides; GenBank accession KP136799) is shown. Each potential open reading frame is illustrated by arrows. The two lines of arrows at the top represent genes on the plus strand, whereas the two lines of arrows at the bottom represent genes on the minus strand. Only herpesvirus genes that were sequenced and found to be homologous are indicated.

exposed cannot be identified serologically, thus the need for virus isolation and molecular analysis. There was also no apparent increase in antibody prevalence with age in these three species. Antibodies in one antibody-positive, wild, male grey seal pup, 1 mo old, likely indicated maternal antibodies (the ELISA detects IgG and does not distinguish between maternal- and pup-derived antibodies). All other antibody-positive YOY harbor and grey seals were likely old enough (>3 mo) to have lost maternal antibodies (King et al. 2001; Goldstein et al. 2004). Because of the possibility of cross-reacting antibodies to other herpesviruses with the ELISA, we used a more-conservative positive cutoff value (>10.0 units/mL) on sera from hooded, harp, and grey seals compared with that for sera from harbor seals (>5 units/mL). Nevertheless, antibody prevalence was high in almost all age-sex classes to one or more unidentified herpesviruses. Antibody concentrations (units/mL) were particularly high in hooded and grey seals. Previous exposure to, and infection with, both an unidentified herpesvirus and PhoHV-2 were detected previously in harp, hooded, and ringed seals. Serum antibodies and viral inclusions in lesions suggested the presence of an unidentified herpesvirus in captive harp seals (Daoust et al. 1994). Antibodies to an unidentified herpesvirus were also detected in sera from adult harp seals (prevalence, 28–37%; $n=183$) from the Barents and Greenland seas and

juvenile to young adult hooded seals (prevalence, 4–7%; $n=100$) from the Greenland Sea (Stuen et al. 1994). Antibodies to PhoHV-2 (prevalence, 50%; $n=4$) were detected in wild ringed seals from Alaska (Zarnke et al. 1997).

Viral isolation and subsequent genome sequencing remain important tools for virus discovery and characterization. Viral isolation and identification reported here indicate that seal gammaherpesviruses can infect at least three seal species in Canada. This is the first report, to our knowledge, of virus isolation and subsequent partial viral genome sequencing to detect novel gammaherpesviruses in hooded, ringed, and harp seals, confirming serologic evidence of exposure to a herpesvirus in hooded and harp seals. Although it is difficult to determine specifically which herpesvirus cross-reactivity was being detected, cross-reactive antibodies can be measured against both alpha- and gammaherpesviruses using the ELISA. Although previous work with Hawaiian monk seals showed a poor correlation between the antibody and testing genome detection by PCR in the same animals, this is not surprising. Serology was the first step in identifying cross-reactive antibodies to an unidentified antigenically related herpesvirus in Hawaiian monk seals, which was later identified as a novel gammaherpesvirus by PCR (Goldstein et al. 2006a).

Most of the reported virus nt sequences in phocid seals are short, partial viral

genome sequences. Furthermore, the herpesvirus glycoprotein B (gB) and DPOL genes are frequently targeted for herpesvirus genomic analyses. Unfortunately, even those two genes are partially sequenced. In addition, these partial gB and DPOL sequences are not overlapping, thus not favoring accurate phylogenomic analyses. The use in the near future of deep genome sequencing will address this shortcoming. To our knowledge, the HaSHV is the first reported extensive phocid seal gammaherpesvirus nt sequence. Based on sequence analyses, 83 putative open reading frames encoding for proteins of at least 100 aa were identified within the HaSHV 108,857 nt sequence, with at least 10 of them being related to well-known herpesvirus genes, such as gB and DPOL (Fig. 3). Protein similarity matrices of those HaSHV putative proteins were computed. High similarities of HaSHV putative proteins (and its genes organization) were found with *Equid herpesvirus 2* aa sequences (data not shown).

The isolated viruses did not appear to be associated with clinical disease other than lethargy in the hooded seal and reduced body condition in both ringed seals (Table 2). Ulcerative lesions associated with gammaherpesvirus infections were reported in Northern elephant seals and Northern and Southern sea otters (Goldstein et al. 2006b; Tseng et al. 2012), but not all gammaherpesviruses detected in marine mammals were associated with lesions or disease. To establish the relationship between the gammaherpesviruses detected in these Canadian phocids and disease (such as viral pneumonia, lymphoma, urogenital tumors, and oropharyngeal ulcers reported in other pinnipeds; Leibich et al. 1994, 2000; Goldstein et al. 2006b; Dagleish et al. 2013), it would be necessary to conduct an epizootiologic and pathologic study of clinically healthy and sick and dead, stranded phocids in Canada. Although most phocid populations in Canadian waters are abundant and not at risk, threats from habitat loss and degradation with chemical and biologic contaminants,

anthropogenic activities, and climate variability, which may affect ice quantity and quality or abundance and quality of prey resources (ice is required in the life cycle of some species), may weaken phocids. Weakened phocids may be more vulnerable to infections, especially opportunistic viruses and those that cause chronic infections and reactivation of latent infections, as with herpesviruses. Since 1994, a sustained temporal decline in body condition of ringed seals in the Amundsen Gulf region has been observed (Harwood et al. 2012). The effect of a herpesvirus infection on the health of individual phocids and their populations over time would be difficult to measure, and at present, negative effects have not been demonstrated.

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