

## Short Communication

# Identification of DNA sequences that imply a novel gammaherpesvirus in seals

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Various herpesviruses have been discovered in marine mammals and are associated with a wide spectrum of disease. In the present study we describe the detection and phylogenetic analysis of a novel gammaherpesvirus, tentatively called phocine herpesvirus 7 (PhHV-7), which was detected in samples collected during an outbreak of ulcerative gingivitis and glossitis from juvenile harbour seals (*Phoca vitulina*) at the Seal Rehabilitation and Research Centre, the Netherlands. The presence of this novel gammaherpesvirus was confirmed by viral metagenomics, while no other viruses other than four novel anelloviruses were detected. However, PhHV-7 DNA was also detected in harbour and grey seals (*Halichoerus grypus*) without gingivitis or glossitis. Genetic analysis of the partial polymerase gene of PhHV-7 detected in both species revealed limited sequence variation. Additional studies are needed to elucidate whether the viruses discovered played a role in the disease observed.

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## MAIN TEXT

Herpesviruses are large, enveloped viruses with a single, linear, double-stranded DNA genome. The order *Herpesvirales* currently consists of three distinct families, *Herpesviridae*, with viruses of mammals, birds and reptiles, *Alloherpesviridae* with viruses of fish and frogs and *Malacoherpesviridae* with viruses of molluscs. The family *Herpesviridae* is further subdivided into subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (ICTV, 2013). In 1985, the first herpesvirus in marine mammals, phocine herpesvirus 1 (PhHV-1), was discovered in harbour seals (*Phoca vitulina*) with pneumonia and hepatitis (Osterhaus *et al.*, 1985). In addition to PhHV-1, more than five herpesviruses are currently described in phocids (Kuiken & Das Neves, 2012; Maness *et al.*, 2011; Venn-Watson *et al.*, 2012). PhHV-1 is to date the only alphaherpesvirus described, while the other five herpesviruses from phocids are classified as gammaherpesviruses.

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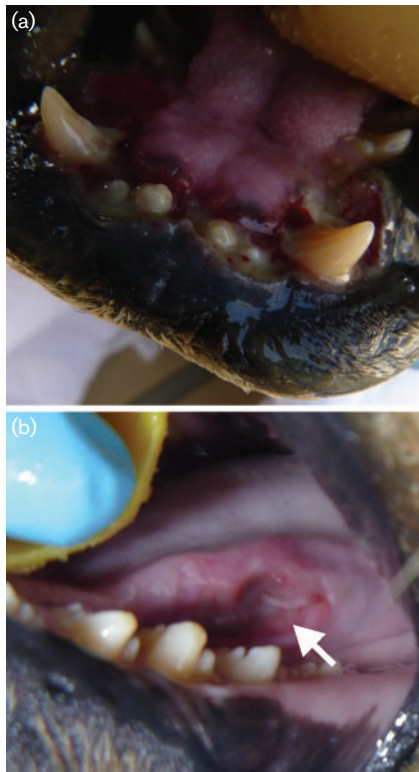
The GenBank/EMBL/DDBJ accession numbers for the genome sequences of phocine herpesvirus and seal anellovirus are KM262781–KM262785 and KP163991–KP163999, respectively.

Three supplementary figures and five supplementary tables are available with the online Supplementary Material.

PhHV-2 was first isolated from leukocytes of harbour seals in 1994 (Harder *et al.*, 1996; Lebich *et al.*, 1994). PhHV-3 was detected in Hawaiian monk seals (*Monachus schauinslandi*), PhHV-4 was detected in northern elephant seals (*Mirounga angustirostris*) with ulcerative lesions of the skin and mucosa, and PhHV-5 and PhHV-6 in harbour seals (Goldstein *et al.*, 2006a, b; Maness *et al.*, 2011).

In the present study, we describe an outbreak of ulcerative gingivitis followed by ulcerative glossitis among harbour seals associated with infection with a novel gammaherpesvirus. During April and May 2014, nine juvenile (between 8 and 10 months of age) harbour seals that were being rehabilitated at the Seal Rehabilitation and Research Centre (SRRC) in Pieterburen, the Netherlands, developed initially diffuse reddening and swelling of the gums in both the upper and lower jaw, mild bleeding, retraction of the gingiva exposing the roots of the teeth, dark staining of the teeth and halitosis (Fig. 1a). In a second stage of the disease, the animals developed lingual ulcerations that were oval, about 10 mm in diameter, 2–4 mm in depth and dark red in colour (Fig. 1b).

Seals that developed gingivitis had initially been admitted to the SRRC during the winter season 2013–2014 due to parasitic pneumonia, but were all successfully treated against



**Fig. 1.** Examples of ulcerative gingivitis and glossitis present in harbour seals. Bleeding gums (a) and an ulcer (arrow) present on the tongue (b).

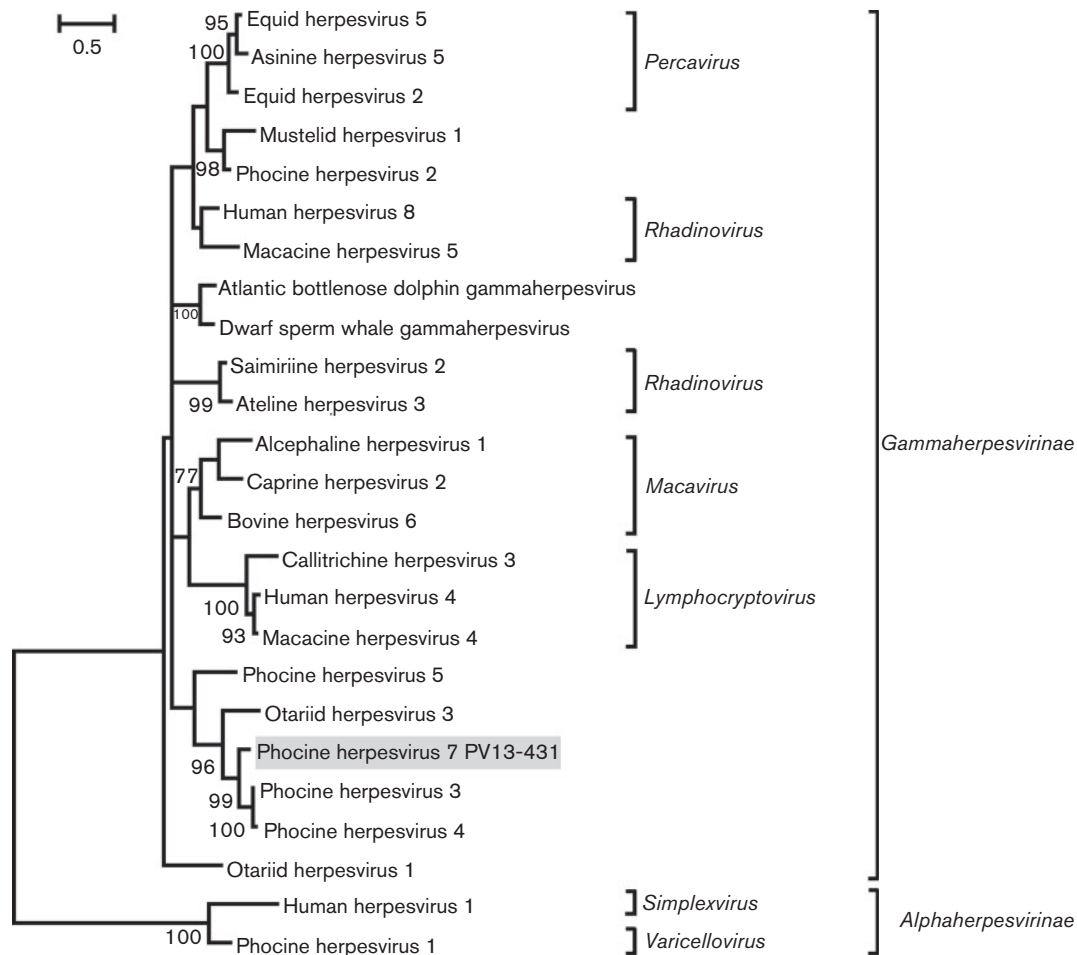
lungworm infection. The seals that developed gingivitis were kept in three different enclosures, although eight animals shared one pool together (Table S1, available in the online Supplementary Material). Seals showing gingivitis and glossitis were empirically treated with anti-inflammatory drugs, prophylactic antibiotic treatment and antiseptic mouthwashes. After 1 week, most of the lesions resolved.

Based on the sudden onset of disease, the observed non-suppurative lesions, the course of the outbreak and absence of apparent problems with water quality and/or nutritional deficiencies, a virus infection was suspected and swab samples were collected from ulcers present on the tongue of affected animals and from the oral mucosa. Swabs were collected in Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U ml<sup>-1</sup> penicillin, 200 µg streptomycin ml<sup>-1</sup>, 100 U polymyxin B sulfate ml<sup>-1</sup>, 250 µg gentamicin ml<sup>-1</sup> and 50 U nystatin ml<sup>-1</sup> (ICN Pharmaceuticals; transport medium) and stored at -70 °C until further processing. Upon defrosting, swab samples were vortexed briefly and nucleic acids were extracted from the supernatant using the High Pure viral nucleic acid kit (Roche). Subsequently, a pan-herpesvirus PCR was performed of which the second PCR was altered by using primers DFA and IYG as described previously (VanDevanter *et al.*, 1996; Venn-Watson *et al.*, 2012). A PCR fragment of

the expected size was obtained from swab samples collected from animals with acute clinical signs. This PCR fragment was ligated into the pCR4 Topo vector (Invitrogen) and sequences of at least three clones in two directions were obtained as described previously (van Leeuwen *et al.*, 2010). Analysis of the sequence fragments obtained by BLAST revealed the presence of a novel gammaherpesvirus, tentatively called phocine herpesvirus 7 (PhHV-7).

To exclude the presence of viral co-infections, sequence-independent RNA and DNA virus screening of four tongue swabs from four different animals, and one oral mucosal swab and one serum sample from one of these animals (Table S2), was performed as described previously (van den Brand *et al.*, 2012; van Leeuwen *et al.*, 2010). Reads obtained were analysed by BLASTN and BLASTX as described previously (Schürch *et al.*, 2014), and sequences most closely related to various genes of gammaherpesviruses were detected in all swabs but not in the serum sample (Table S2). In addition, sequences that were most closely related to anelloviruses were detected in the serum sample and in two swabs, while no other viral sequences were detected. Based on reads obtained by next-generation sequencing, additional specific primers were designed and the partial polymerase gene (1026 nt; GenBank accession number KM262784) of PhHV-7 was obtained from seal PV13-431 as described previously (van Leeuwen *et al.*, 2010). Alignment and phylogenetic analysis of the partial DNA polymerase gene of PhHV-7 PV13-431 with various other gammaherpesviruses using MAFFT version 7 (Kato & Standley, 2013) and MEGA6 (Tamura *et al.*, 2013) indicated that this virus was most closely related to PhHV-3, with pairwise identities of 75% at the nucleotide level and 86% at the amino acid level (Fig. 2, Table S3). According to the criteria of the International Committee on Taxonomy of Viruses (ICTV) (Pellett *et al.*, 2012), a novel herpesvirus species should have a distinct genome and epidemiological or biological characteristics. Since there was considerable sequence difference between PhHV-7 and the most closely related viruses (PhHV-3 and PhHV-4), and these viruses were detected in different species of seals, PhHV-7 may be classified as a novel species. However, only limited sequence information is available for most herpesviruses detected in marine mammals, and additional sequence information is necessary to further understand the phylogenetic relationships of these viruses.

Of interest, gammaherpesviruses have been associated with ulcerative lesions of the skin and mucosa in a number of animal species, among which are several carnivore species including northern sea otters (*Enhydra lutris kenyoni*), California sea lions (*Zalophus californianus*), northern elephant seals (*Mirounga angustirostris*) and a fisher (*Martes pennanti*) (Gagnon *et al.*, 2011; Goldstein *et al.*, 2006b; Smith *et al.*, 2008; Smolarek Benson *et al.*, 2006; Tseng *et al.*, 2012; Venn-Watson *et al.*, 2012). In humans infected with human immunodeficiency virus, human herpesvirus 4 (Epstein-Barr virus) can cause oral hairy leukoplakia and both human herpesviruses 4 and 8 were detected in oral ulcers (Di Alberti



**Fig. 2.** Phylogenetic analysis of the partial polymerase gene of PhHV-7. The amino acid sequence of the partial polymerase gene of PhHV-7 detected in PV13-431 was aligned with various other herpesviruses. Subsequently, an LG (Le and Gascuel) + G + I tree was built by analysis with PROTEST 3.0 (Darriba *et al.*, 2011) based on an alignment of 450 nt in MEGA6 with 500 bootstrap replicates. Only bootstrap values >70 are shown. GenBank accession numbers: alcephaline herpesvirus 1, AF005370; asinine herpesvirus 5, KC825357; ateline herpesvirus 3, AF083424; atlantic bottlenose dolphin gammaherpesvirus, AY952776; bovine herpesvirus 6, KJ705001; caprine herpesvirus 2, HQ116812; dwarf sperm whale gammaherpesvirus, AY949830; equid herpesvirus 2, NC\_001650; equid herpesvirus 5, GQ325597; human herpesvirus 1, P04293; human herpesvirus 4, NC\_007605; human herpesvirus 8, U93872; macacine herpesvirus 4, NC\_006146; macacine herpesvirus 5, NP\_570750; mustelid herpesvirus 1, AF376034; otariid herpesvirus 1, AF236050; otariid herpesvirus 3, JX080682; phocine herpesvirus 1, U92269; phocine herpesvirus 2, GQ429152; phocine herpesvirus 3, DQ093191; phocine herpesvirus 4, DQ183057; phocine herpesvirus 5, GQ429153; phocine herpesvirus 7, KM262784; saimiriine herpesvirus 2, X64346.

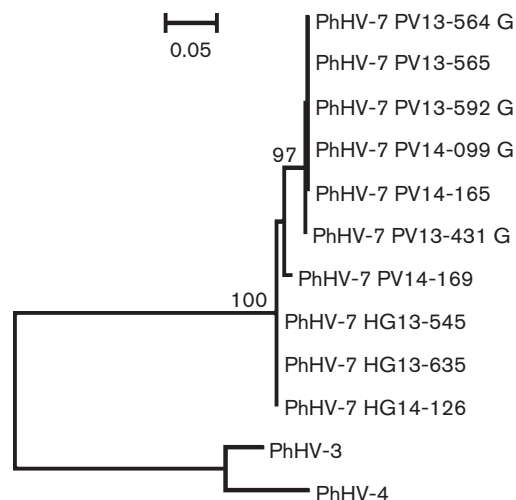
*et al.*, 1997; Hille *et al.*, 2002; Syrjänen *et al.*, 1999). Although gammaherpesviruses are generally considered lymphotropic and are often associated with lymphoproliferative disease (Ackermann, 2006), these studies suggested that infection with certain gammaherpesviruses, in combination with a stressor or underlying disease, can induce the development of ulcerative lesions. To elucidate the putative role of PhHV-7 in the ulcerative gingivitis and glossitis observed, and to determine its prevalence among seals present in the SRRC, oral mucosal swabs were collected from all seals with gingivitis ( $n=9$ ) and two additional control groups with no

signs of gingivitis (Table S1) and tested by use of a PhHV-7-specific PCR. The first control group consisted of 14 harbour seals in the same age range, and reason for stranding, as the group with gingivitis. Two of these animals were kept in the same enclosure as the animals with gingivitis, while the others were kept in other enclosures. The second control group consisted of eight juvenile grey seals (*Halichoerus grypus*) that were present at the SRRC at the same time the outbreak of gingivitis occurred. These grey seals were held in other enclosures than the group with gingivitis. Nucleic acids extracted from the swabs were tested for the presence

of PhHV-7 DNA by PCR using forward primer GGATTACTGGAGTATCTGGAG and reverse primer CTGTCAGAGATTCTAGGGTTTC to amplify a fragment (138 nt) of the partial polymerase gene of PhHV-7. Nucleic acids extracted from transport medium were used as a negative control in the PCR. A PCR fragment of the expected size was detected in seven of nine swab samples collected from the animals with ulcerative gingivitis, while PhHV-7 DNA was also detected in 12 of 14 swab samples collected from the harbour seal control group and in seven of eight swab samples collected from the grey seal control group. Sequence analysis of a proportion of the PCR products obtained indeed confirmed the presence of PhHV-7 DNA. The persistence of PhHV-7 infection was evaluated by testing swab samples collected from four harbour seals that were still present at the SRRC five weeks after the outbreak with gingivitis (Table S1). In two of the four animals tested, PhHV-7 DNA was detected by PCR, which showed that PhHV-7 remained present in seals at the SRRC for more than 1 month.

To elucidate whether sequence variation exists between PhHV-7 detected in grey seals and harbour seals, the nucleotide sequences of the partial polymerase gene (816 nt) of PhHV-7 detected in six harbour seals (PV13-564, PV13-592, PV13-565, PV14-099, PV14-165, PV14-169) and three grey seals (HG13-545, HG13-635, HG14-126) were obtained by PCR using forward primer CTGCTACTCCACCATGATTTTCAG and reverse primer GTGAGGTCTGATATTTCCACGTG. Alignment and phylogenetic analysis of the PCR fragments obtained indeed revealed variation in the nucleotide and deduced amino acid sequence of PhHV-7, not only in harbour and grey seals but also within PhHV-7 detected in harbour seals (Figs 3 and S1). Pairwise identities on the nucleotide level between PhHV-7 in grey and harbour seals ranged between 97.3 and 98.6%, while pairwise identities at the amino acid level ranged between 98 and 98.9%. Of interest, sequence variation between other DNA viruses (phocine herpesvirus 1 and seal parvovirus) detected in grey and harbour seals suggested that the metapopulation of harbour and grey seals acts as a single reservoir for these viruses (Bodewes *et al.*, 2014; Martina *et al.*, 2002). Furthermore, it has been demonstrated for other gammaherpesviruses isolated from harbour and grey seals that these are able to replicate in cells from other species *in vitro* (Martina *et al.*, 2007). Additional experiments, including sequencing of other genes of PhHV-7, are necessary to elucidate the biological impact of the genetic variation observed.

To evaluate the presence of this novel herpesvirus among free-ranging seals, oral mucosal swabs were collected from 16 harbour seal pups (less than 2 months of age) upon admission to the SRRC in 2014, but PhHV-7 DNA was not detected in any of those swab samples. The historic presence of this novel herpesvirus in harbour seals was evaluated in ulcers present in the mouth ( $n=8$ ) or on the skin ( $n=4$ ) of 10 harbour seals that died in 2001 and 2002 (Table S5). Samples of those ulcers had been collected at



**Fig. 3.** Phylogenetic analysis of PhHV-7 detected in harbour and grey seals. The nucleotide sequences of the partial polymerase gene of PhHV-7 detected in these seals were aligned with phocine herpesviruses 3 and 4, and a HKY + G (Hasegawa–Kishino–Yano) tree was built as determined by JModelTest 2.1.3 (Darriba *et al.*, 2012) based on an alignment of 409 nt in MEGA6 with 500 bootstrap replicates. Only bootstrap values  $>70$  are shown. PhHV-7 detected in animals with ulcerative gingivitis and glossitis is indicated with a G. GenBank accession number: phocine herpesvirus 3, DQ093191; phocine herpesvirus 4, DQ183057; phocine herpesvirus 7 PV13-431, KM262784; phocine herpesvirus 7 PV13-564, KP163991; phocine herpesvirus 7 PV13-565, KP163992; phocine herpesvirus 7 PV13-592, KP163993; phocine herpesvirus 7 PV14-099, KP163994; phocine herpesvirus 7 PV14-165, KP163995; phocine herpesvirus 7 PV14-169, KP163996; phocine herpesvirus 7 HG13-545, KP163997; phocine herpesvirus 7 HG13-635, KP163998; phocine herpesvirus 7 HG14-126, KP163999.

autopsy and stored at  $-70^{\circ}\text{C}$ . They were defrosted and homogenized as described previously (Bodewes *et al.*, 2013b), and nucleic acids extracted were tested for the presence of PhHV-7 DNA. PhHV-7 DNA was detected in five samples; two were from mouth ulcers and three from skin ulcers (Table S5). However, subsequent histopathological evaluation of duplicate samples of one ulcer each on the skin and the oral mucosa in which PhHV-7 DNA was detected showed no intranuclear inclusions (Fig. S2).

In addition to this novel herpesvirus, the near complete genomes of two novel anelloviruses, tentatively called seal anelloviruses (SeAv) 4 and 5 (SeAv4, 2223 nt, GenBank accession KM262783; SeAv5, 2056 nt, GenBank accession KM262782), and the complete genomes of two other novel anelloviruses, tentatively called SeAv6 and SeAv7 (SeAv6, 2143 nt, GenBank accession KM262785; SeAv7, 2165 nt, GenBank accession KM262781), were obtained by assembly of reads from the deep sequencing data for serum of PV13-431. The genome organizations of the two partially sequenced anelloviruses are similar to a torque tenovirus



detected in a pine marten (MmTTV1) (van den Brand *et al.*, 2012), while those of the two completely sequenced anelloviruses were similar to anelloviruses previously detected in seals, with three major ORFs and a TATA-box (Bodewes *et al.*, 2013a; Ng *et al.*, 2011) (data not shown). Alignment and phylogenetic analysis of the amino acid sequences of ORF1 of SeAv4-7 with various other anelloviruses, using CLUSTAL OMEGA (Sievers *et al.*, 2011) and MEGA6 (Tamura *et al.*, 2013), revealed that SeAv4 and SeAv5 were most closely related to each other (65% identity at the nucleotide level and 64% at the amino acid level) and to MmTTV1 (46% identity at the nucleotide level and 32–34% at the amino acid level). SeAv6 was most closely related to SeAv3 and SeAv7 (67–68% identity at the nucleotide level and 60% at the amino acid level), while SeAv7 was also very closely related to SeAv3 (94% identity at the nucleotide level and 97% at the amino acid level) (Fig. S3, Table S4). Genera within the *Anelloviridae* are currently defined as having less than 44% identity at the nucleotide level in ORF1, and species are defined as having less than 65% identity at the nucleotide level in ORF1 (ICTV, 2013). This indicates that SeAv 4 and 5 belong to a novel species within a putative novel genus that currently consists solely of MmTTV1. SeAv6 and SeAv7 belong to the same species as SeAv3. Anelloviruses have not been associated with ulcerative lesions in humans and animal species (Hino & Miyata, 2007; Ng *et al.*, 2011; Toyoda *et al.*, 2000), and additional studies are necessary to elucidate the role of these viruses in the lesions observed in these harbour seals.

In conclusion, a novel herpesvirus and four novel anelloviruses were identified during an outbreak of ulcerative gingivitis and glossitis among harbour seals. Additional studies are necessary to understand the pathogenesis and epidemiology of infections with PhHV-7 and other gammaherpesviruses in various species of seal.

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