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BRIEF REPORT

Identification of a novel gammaherpesvirus associated with (muco)cutaneous lesions in harbour porpoises (*Phocoena phocoena*)

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Abstract Herpesviruses infect a wide range of vertebrates, including toothed whales of the order *Cetacea*. One of the smallest toothed whales is the harbour porpoise (*Phocoena phocoena*), which is widespread in the coastal waters of the northern hemisphere, including the North Sea. Here, we describe the detection and phylogenetic analysis of a novel gammaherpesvirus associated with mucocutaneous and skin lesions in stranded harbour porpoises along the Dutch coast, tentatively designated phocoenid herpesvirus 1 (PhoHV1). Phylogenetically, PhoHV1 forms a monophyletic clade with all other gammaherpesviruses described in toothed whales (*Odontoceti*) to date, suggesting a common evolutionary origin.

Herpesviruses are double-stranded DNA viruses with a typical virion morphology, consisting of an icosahedral nucleocapsid that is surrounded by a tegument and a host-derived envelope [1]. Herpesviruses infect a wide range of vertebrates and are generally host-specific [2]. Over long periods of time, herpesviruses have co-evolved with their natural hosts and, as such, are well adapted to them and generally cause only mild disease [3]. All herpesviruses are taxonomically classified as members of the order *Herpesvirales*, which consists of three families [4]. The family *Herpesviridae* comprises the mammalian, avian and reptilian herpesviruses, the family *Alloherpesviridae* comprises the amphibian and piscine herpesviruses, and the family *Malacoherpesviridae* comprises invertebrate herpesviruses. Members of the family *Herpesviridae* are by far the best characterized, and this family is further subdivided into the subfamilies *Alpha-*, *Beta-*, and *Gammaherpesvirinae*.

The first herpesvirus infecting a marine mammal was phocid herpesvirus 1, which was found in harbour seals (*Phoca vitulina*) in the Netherlands [5]. To date, a total of seven herpesviruses have been described for phocids: one alphaherpesvirus and six gammaherpesviruses [6]. The first detection of a herpesvirus in cetaceans dates back to the late 1980s, when herpesvirus-like particles were observed by electron microscopy (EM) in skin lesions in beluga whales (*Delphinapterus leucas*) in Canada [7, 8] and in the brain of a harbour porpoise (*Phocoena phocoena*) stranded in Sweden in 1988 [9]. Ever since, herpesviruses in toothed whales of the parvorder *Odontoceti* have been associated with skin lesions [7, 8, 10–14], genital lesions [10, 15–20] and oral lesions [10, 16]. Occasionally, alphaherpesviruses have been found to cause encephalitis [9, 11] interstitial nephritis [21], or a fatal systemic infection [22–24] in cetaceans. Here, we report a novel gammaherpesvirus associated with mucocutaneous and skin lesions in harbour porpoises stranded on the Dutch coast.

Harbour porpoises are small cetaceans that inhabit coastal waters and shelf seas of the North Atlantic and the North Pacific oceans [25]. They are the most prevalent cetaceans in the North Sea, with an estimated population of

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250,000 individuals, of which 85,000 may inhabit Dutch waters [26–28]. Since 2008, harbour porpoises that stranded dead, or died shortly after stranding on the Dutch coast, are subjected to postmortem investigation on a regular basis [29].

In August 2012, an adult female harbour porpoise was found stranded and dying on the island of Texel and was subsequently necropsied under the identification number UT775. At necropsy, lesions were present in the skin and the mucocutaneous junctions of the genital slit and upper jaw, ranging in size from 10 to 60 mm in diameter (Fig. 1A and B). These varied from round to oval, with protruding margins and an indented centre; occasionally, necrosis was visible on the cut surface. In addition, oesophageal ulcerations and severe bronchopneumonia were observed. Tissue samples from various organs were fixed in 4 % phosphate-buffered formalin, embedded in paraffin, cut into 4-µm sections, and stained with haematoxylin and eosin. The acanthotic epidermis showed focal superficial erosions with bacteria. The superficial, hyperplastic epithelial cells were swollen with pale, eosinophilic material in the cytoplasm, and nuclei showed chromatin margination and eosinophilic intranuclear inclusion bodies that were surrounded by a halo (INI, Fig. 1C). Small numbers of lymphocytes and some neutrophils were present in the superficial dermis. There was mild focal epidermal necrosis with more-extensive, predominantly non-suppurative infiltrates and a few multinucleated giant cells. Other findings included stomatitis, severe multifocal to coalescing broncho-interstitial and pyogranulomatous pneumonia with few intralesional nematode larvae (*Pseudalius inflexus*) and mild chronic multifocal lymphoplasmacytic meningoencephalitis. The non-suppurative infiltrates and INI were suggestive of a viral infection.

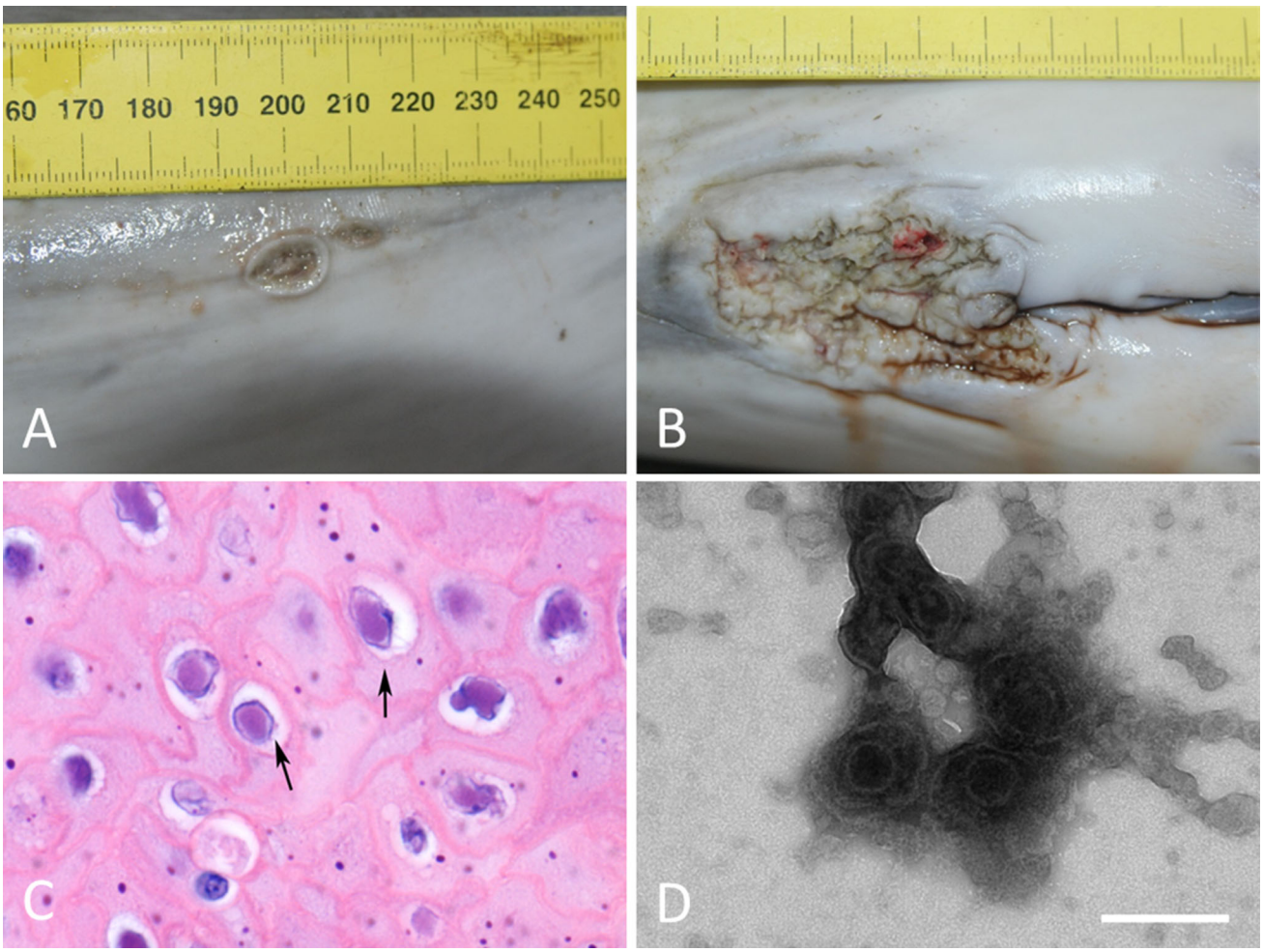


Fig. 1 Lesions associated with phocoenid herpesvirus 1. (A) Skin lesion in the abdominal area. Note the elevated margins and dented centre. The ruler markings are in millimetres. (B) Skin lesion cranial to the genital slit. The margins are elevated, the dented centre has an very irregular surface with focal necrosis and haemorrhage. The ruler markings are in millimetres. (C) Photomicrograph of the epidermis with eosinophilic intranuclear inclusion bodies (arrows). H&E stain, magnification is x 80. (D) Electron microscopic image of a negatively stained tissue suspension from the genital lesion shown in B. Several intact and disrupted herpesvirus-like particles are visible. The scale bar is 200 nm

Selected tissues were examined by transmission electron microscopy for the presence of viral particles. Frozen (-80 °C) tissue samples of 0.5 g from the genital lesion shown in Fig. 1B, the cerebrum and the cerebellum were mechanically lysed on ice in 2 ml of cold PBS using a Dounce homogenizer. The organ suspensions were further diluted in PBS and cleared by centrifugation (2000 rpm for 10 min at 4 °C), passed through a 70-µm cell strainer, further cleared by centrifugation (20 min at 4000 rpm at 4 °C), and passed through a sterile 1.2-µm filter. The cleared supernatant was concentrated by ultracentrifugation through a 20 % sucrose cushion (25,000 rpm for 2.5 h at 4 °C), and the visible pellets were resuspended in PBS. Samples were negatively stained with a 1 % ammonium molybdate solution. Imaging was performed using an FEI Tecnai 12 electron microscope at 80 kV. Particles consistent with the size and morphology of a herpesvirus (a capsid approximately 100 nm in diameter, surrounded by an envelope 150-200 nm in diameter) were present in the sample from the genital lesion (Fig. 1D).

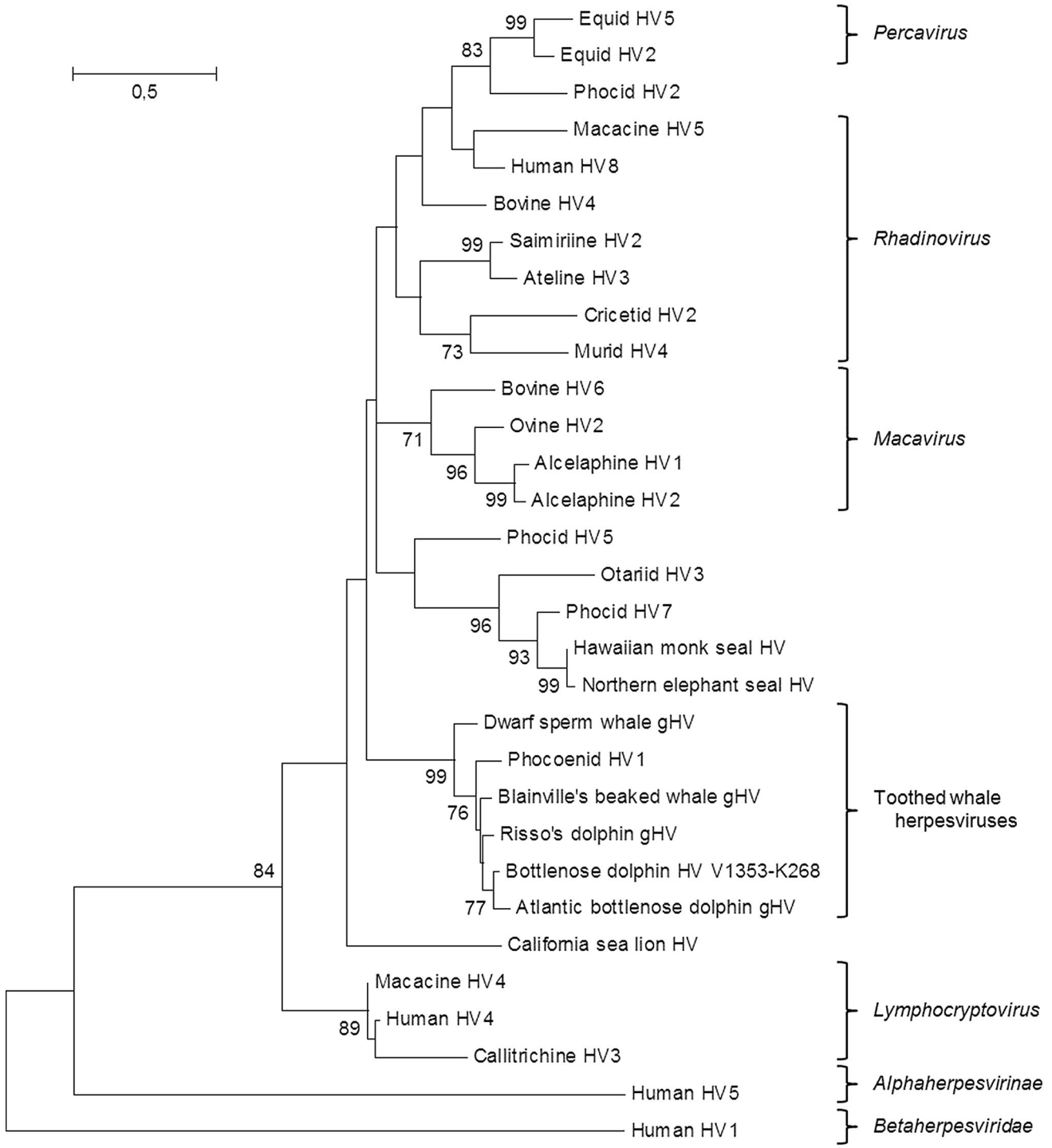
To characterize the observed herpesvirus at the molecular level, DNA was extracted from deep-frozen tissue samples of harbour porpoise UT775 using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's protocol. A nested pan-herpesvirus PCR targeting the polymerase gene was performed as described previously [30]. Fragments of the expected sizes were amplified from DNA from the genital lesion shown in Fig. 1B, and after the first round of PCR, amplicons were excised from the gel using a QIAquick Gel Extraction Kit (QIAGEN), ligated into the pCR2.1-TOPO vector (Life Technologies), cloned into One Shot TOP10 chemically competent *E. coli* cells (Life Technologies), and sequenced from both ends by the Sanger method (Seqlab - Sequence Laboratories Göttingen). BLAST searches indicated that the sequence belonged to a novel cetacean gammaherpesvirus, tentatively designated phocoenid herpesvirus 1 (PhoHV1) (GenBank accession number KT591613).

The sequence of a portion of the DNA polymerase gene of PhoHV1 (685 nt) was determined, and this partial sequence was translated and aligned, using ClustalX2 [31], with available sequences of other marine mammal gammaherpesviruses, with a minimal overlapping sequence length of 65 %, 16 reference sequences representing the four gammaherpesvirus genera, and human herpesvirus 1 and 5 as outliers representing the alpha- and betaherpesviruses, respectively. Phylogenetic analysis using MEGA6.06 [32] indicated that PhoHV1 groups with all other toothed whale gammaherpesviruses described so far, including two bottlenose dolphin gammaherpesviruses, dwarf sperm whale gammaherpesvirus, Blainville's beaked whale gammaherpesvirus and Risso's dolphin gammaherpesvirus (Fig. 2). This is supported by pairwise nucleotide sequence comparisons, with the highest identity of 82.2 % (562/684 nt) with Blainville's beaked whale gammaherpesvirus (AY949828.1).

We used PCR to investigate the occurrence of PhoHV1 in frozen tissue samples from skin lesions of harbour porpoises stranded on the Dutch coast between 2010 and 2013. Skin lesions that were likely to have been caused by grey seal predation or fisheries bycatch [29] were excluded from analysis. DNA was extracted from a total of 98 deep-frozen skin lesions of 60 animals, using a QIAamp DNA Mini Kit. PCR was performed using Taq DNA polymerase (Thermo Fisher Scientific) with a set of primers specifically targeting a conserved region of the DNA polymerase gene: forward primer PhphHV.DNApol.F23 (5'-AGTGCTGCTAGTTACGGAGG-3') and reverse primer PhphHV.DNApol.R23 (5'-CAACAGCTCGCCATCAAAGT-3'). The thermal profile of the PCR consisted of a denaturation step of 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 5 min, and the reaction was carried out in a T100 Thermal Cycler (Bio-Rad). PCR products were analysed by gel electrophoresis in order to identify fragments of the expected sizes (324 bp).

A total of 10 skin or mucocutaneous lesions of five porpoises (including UT775) tested positive: four adult females and one adult male. Histopathological examination of the skin lesions of four of these animals indicated dermatitis with INI. Examination of the internal organs of 3 of the animals showed oesophagitis or oesophagus ulceration, with INI in two animals. The oesophageal mucosal lesions seen in these animals were severe enough to negatively affect food intake, potentially leading to hypoglycaemia, dehydration, undercooling and eventually death. Excluding the male harbour porpoise, which probably died as a result of bycatch, the nutritional condition of the female PhoHV1-positive animals was normal to very poor.

The host range of herpesviruses of the subfamily *Gammaherpesvirinae* is restricted to the family or order of the natural host [1]. Usually, they specifically infect lymphocytes, resulting in lymphoproliferative malignancies. Latency is normally established in lymphocytes or lymphoid tissue. In contrast, all toothed whale gammaherpesviruses associated with pathological changes were detected in skin and mucosal lesions [10, 12, 15, 17–20]. Similarly, several gammaherpesviruses described in seals have also been associated with skin and mucosal ulcerations (summarized in reference [6]). Further research is warranted to determine whether these marine mammal gammaherpesviruses primarily cause the observed skin and mucosal lesions or whether these lesions are secondary to a lymphoproliferative disorder, which is commonly observed in most other gammaherpesvirus infections.



A herpesvirus is assigned to a separate species if it has distinct epidemiological or biological characteristics and a distinct genome that represents an independent replicating lineage [2]. Herpesvirus species whose members are closely related are grouped together in the same genus. The six toothed whale gammaherpesviruses described so far infect an evolutionarily related group of animal species (*Odontoceti*), are all associated with skin or mucosal lesions in contrast to most other gammaherpesviruses infecting non-cetaceans, and form a monophyletic clade based on partial DNA polymerase gene sequence analysis [33 and this study]. Hence, we propose that all toothed whale gammaherpesviruses should be assigned to a yet to be established gammaherpesvirus genus, tentatively named

◀**Fig. 2** Phylogenetic analysis of the partial amino acid sequence of the DNA polymerase gene of phocoenid herpesvirus 1 (GenBank accession number KT591613). The alignment included available sequences of other marine mammal gammaherpesviruses (phocid herpesvirus 2, ACV86607.1; phocid herpesvirus 5, ACV86608.1; otariid herpesvirus 3, AFP23381.1; phocid herpesvirus 7, AJA71665.1; Hawaiian monk seal herpesvirus, AAY90140.1; northern elephant seal herpesvirus, ABA26922.1; dwarf sperm whale gammaherpesvirus, AAX47053.1; Blainville's beaked whale gammaherpesvirus, AAX47051.1; Risso's dolphin gammaherpesvirus, ABC33905.1; bottlenose dolphin herpesvirus V1353-K263, AAX55677.2; Atlantic bottlenose dolphin gammaherpesvirus, AAX55676.1; California sea lion herpesvirus, AF236050.1), 16 reference sequences representing the four gammaherpesvirus genera (equid herpesvirus 5, YP_009118399.1; equid herpesvirus 2, NP_042605.1; macacine herpesvirus 5, NP_570750.1; human herpesvirus 8, YP_001129355.1; bovine herpesvirus 4, NP_076501.1; saimiriine herpesvirus 2, NP_040211.1; ateline herpesvirus 3, NP_047983.1; cricetid herpesvirus 2, YP_004207849.1; murid herpesvirus 4, NP_044849.1; bovine herpesvirus 6, YP_009041990.1; ovine herpesvirus 2, YP_438136.1; alcelaphine herpesvirus 1, NP_065512.1; alcelaphine herpesvirus 2, YP_009044396.1; macacine herpesvirus 4, YP_068007.1; human herpesvirus 4, YP_001129507.1; callitrichine herpesvirus 3, NP_733857.1), and human herpesvirus 1 and 5 as outliers representing the alpha- and betaherpesviruses (human herpesvirus 5, YP_081513.1; human herpesvirus 1, NP_044632.1). The alignment was trimmed to the available residues of phocoenid herpesvirus 1, resulting in a total number of 156 included positions, at a partial deletion of 92 %. The unrooted maximum-likelihood tree was constructed using MEGA 6.06 with the best-fit substitution model (LG+G) with 500 bootstrap replicates. Bootstrapping values >70 % are indicated at the nodes. The scale bar indicates the number of substitutions per site

"*Toowhavirus*" (too from toothed, wha from whale). Additional sequence data, as well as identification of novel whale herpesviruses in the future, are required to further develop our understanding of the evolution of gammaherpesviruses infecting cetaceans.

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