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Research paper

Herpesvirus infection in marine mammals: A retrospective molecular survey of stranded cetaceans in the Portuguese coastline

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A B S T R A C T

Herpesvirus (HVs) infection has already been reported in cetaceans, but available information on its epidemiology is scarce. In this study we surveyed a total of 179 cetaceans belonging to 6 different species. Samples were obtained from cetaceans stranded along the Portuguese coastline, belonging to populations that roam the north-east region of the Atlantic Ocean. Detection of HVs was performed by conventional nested PCR. Amplicons were sequenced by Sanger's method and sequences used to construct phylogenetic trees by Maximum Likelihood method. Our results show that prevalence of positive samples, among fresh carcasses, reached 14.3% (10/70) and both alpha and gammaherpesvirus were detected. Histopathology showed that herpesvirus infection varied from absence of signs compatible with disease, localized genital lesions and systemic disease. Phylogenetic analysis revealed three clusters within the alphaherpesvirus family; within the gammaherpesvirus no subdivision was detected. All clusters included animals from different species and geographic origins. In seven of the positive HVs samples, co-infections with other agents such as morbillivirus and *toxoplasma gondii* were detected. The viral nucleotide sequences were not assigned to a specific animal species, nor presented a given geographic distribution, which may imply a wider distribution of herpesvirus in these animal populations. Our results are also the first report of herpesvirus infection in common dolphins (*Delphinus delphis)*, with both alpha and gammaherpesvirus detected.

1. Introduction

Herpesviruses (HVs) are responsible for a wide range of diseases and the course of infection can vary from subclinical to fatal infections with localized or systemic lesions (Davison, 2008; Saliki et al., 2006). Their most unique feature is the persistent infection interleaved with periodic or continuous shedding (MacLachlan and Dubovi, 2011a). The viral genome can remain latent, either as an episome or integrated in the host genome (provirus). Upon viral genome reactivation, often promoted by stress cellular signals, lytic viral replication takes place in the nucleus of the infected cell during the active stage of infection. The family Herpesviridae comprises three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (ICTV, 2011).

The characteristic appearance of HVs was initially used as an identification method to assign them to the taxonomic family *Herpesviridae*. Later, they were allocated to each subfamily by biological criteria (McGeoch et al., 2006). With the advent of molecular biology techniques such as the polymerase chain reaction (PCR), genetic criteria allowed the characterization of these viruses and their taxonomic assignment.

Research in this area rapidly evolved, due to the amount of HVs described: by 1996 around 100 distinct HVs had been described (Vandevanter et al., 1996); 120 in 2002 (Davison, 2002) and, most recently, over 200 HV have been reported (Pellett and Roizman, 2013). The growing number of known HVs sequences is mostly due to the development of a pan-herpesvirus detection system (Vandevanter et al., 1996). This system was designed to target the conserved motifs on the viral DNA polymerase (Dpol) using degenerate primers, allowing the amplification of a vast array of distinct HVs from different host species. This approach has allowed the successful investigation of the presence of HVs in different species, from pigeons and horses (Ehlers et al., 1999) to reptiles (Wellehan et al., 2003) and bats (Molnár et al., 2008), allowing the virus identification and the taxonomic assignment to a subfamily (α, γ or β).

In marine mammals, HVs were first reported in harbor seals (Phocine herpesvirus – 1 [PhHV-1]) by electron microscopy and serum neutralization assays (Osterhaus et al., 1985). Since then, HVs have been reported in several species of pinnipeds (Goldstein et al., 2006a,

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2006b; Harder et al., 1996; Lipscomb et al., 2000). In odontocete cetaceans HVs have already been detected in Monodontidae (e.g. belugas, Martineau et al., 1988), Phocoenidae (porpoises, Kennedy et al., 1992), Delphinidae (dusky dolphins and striped dolphins, Van Bressem et al., 1994, Bellière et al., 2010) and Ziphiidae, (beaked whales, Arbelo et al., 2012, 2010). In Mysticetes, HVs have been recently reported in two species from the *Balaenoptera* genus (Melero et al., 2015).

With respect to cetaceans, viruses capable of causing disease and immunosuppression, such as morbilliviruses, were detected in animals with concurrent herpesvirus infection (Bellière et al., 2010). Also, papillomaviruses were detected in herpesvirus positive animals (Cruz et al., 2014). So far, cetacean HVs were included in the *Alphaherpesvirinae* or *Gammaherpesvirinae* subfamilies. Alphaherpesviruses have been associated with fatal disseminated infections (Blanchard et al., 2001), localized genital lesions (Smolarek Benson et al., 2006), proliferative dermatitis (Manire et al., 2006), tubule-interstitial nephritis (Arbelo et al., 2012), among other infections (Arbelo et al., 2010; Sierra et al., 2014; Soto et al., 2012). Gammaherpesviruses have been detected in cetaceans associated to cutaneous and mucosal lesions (Saliki et al., 2006; Smolarek Benson et al., 2006).

2. Theory

So far, no information on herpesvirus infection was available for samples obtained in Portugal. Scientific reports of herpesvirus infection in several species of cetaceans on samples obtained in Spain are available, but mostly from samples from the Canary Islands and Mediterranean (Arbelo et al., 2012, 2010; Bernaldo De Quirós et al., 2011; Melero et al., 2015; Sierra et al., 2015). No information is available from the populations that roam the Atlantic waters of the Iberian Peninsula.

Previous work identified the presence of morbillivirus infection in samples from the Portuguese coastline (Rocha De Medeiros Bento et al., 2016). Integrating this information with a survey of herpesvirus will allow us to deepen the knowledge of the health status of these populations.

The present study aims at identifying novel or known herpesviruses in samples from cetaceans stranded on the Portuguese coastline. Phylogenetic relations between these new herpesviral sequences and representatives of the known Herpesviridae subfamilies and genera will be explored through Maximum Likelihood (ML) analyses. In available samples, histopathology will be performed in order to identify lesions compatible with herpesviral infection, and to assess cause of death.

3. Materials and methods

3.1. Sampling

The animals stranded along the coast of Portugal between 2011 and 2014 and were accessed by experienced personnel belonging to the Portuguese stranding network, coordinated by the Institute for Nature Conservation and Forests (ICNF) and Sociedade Portuguesa de Vida Selvagem (SPVS) (Fig. 1a and b).

Detailed necropsies were performed, depending on the carcasses' preservation status (decomposition code) (Geraci and Lounsbury, 1993) and the cause of death was determined when possible. Data and samples were collected according to standard protocols (Kuiken and Hartmann, 1991) and each animal was identified using an alphanumeric code composed of the initials for the species, an attributed number, and the year of stranding. Samples were kept in 2 mL vials, submerged in RNALater (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20 °C, until the molecular analysis. For histology, tissue samples were stored in 10% formalin. All the technicians that work on marine animal strandings from the Portuguese Wildlife Society are licensed for capture, handling, tagging and sample collection in mainland Portugal under the Decree-Law n° 140/99 of 24th April, with new redaction given by Decree-Law n° 49/2005 of 24th February and Decree-Law n° 316/89 of 22nd September. The licenses are issued by the Institute of Nature Conservation and Forests. These licenses are issued every year. Also, The Portuguese Wildlife Society, which holds the Marine Animal Tissue Bank, is registered in CITES as a scientific institution with code PT009. Data and samples were collected according to standard protocols (Kuiken and Hartmann, 1991) and each animal was identified using an alphanumeric code composed of the initials for the species, an attributed number, and the year of stranding.

A total of 179 stranded cetaceans belonging to 6 different species were tested for the presence of herpesvirus DNA (Table 1): 126 common dolphins (*Delphinus delphis*), 31 harbor porpoises (*Phocoena phocoena*), 17 striped dolphins (*Stenella coeruleoalba*), 2 bottlenose dolphins (*Tursiops truncatus*), 1 long-finned pilot whale (*Globicephala melas*) and 2 pygmy sperm whales (*Kogia breviceps*). Samples identification included an alphanumeric code composed of the initials for the species, an attributed number, and the year of stranding. Of the animals tested 76 were males and 100 females. In three animals the sex was not registered. Most samples were from juvenile animals (115), while only 52 were adult animals. Animals less than one year old summed to a total of 11 animals. In one animal, age class was not estimated.

3.2. DNA extraction

For each tested animal, DNA was extracted from a pool of organ tissue containing, when available, liver, kidney, lung and brain samples. If present, samples of genital lesions were also included in the pool. Total DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Dusseldorf, Germany), according to the manufacturer's protocol. DNA concentration was measured with Nanodrop 2000C (Thermo Scientific, Whaltam, MA, USA), and stored at −20 °C.

3.3. DNA amplification

Presence of herpesvirus DNA in extracted total DNA was detected by PCR, in a nested format, using five degenerate primers (Vandevanter et al., 1996). This technique targets a conserved region within the herpesvirus polymerase gene, with an expected amplicon size ranging from 215 bp to 315 bp.

In the first PCR reaction, 100 ng of extracted total DNA were mixed with 5 PRIME MasterMix (5PRIME GmbH, Hilden, Germany) (1.6 units of Taq DNA polymerase; 250 μM of each dNTP; 2.0 mM of Mg2 +) and 400 nM of each primer (DFA, ILK and TGV) in a final volume of 25 μL. The PCR product obtained in the first reaction was used as a template for the second round PCR (2.5 μL), using the same mastermix and 400 nM of primers TGV and IYG.

Cycling conditions for both PCR reactions included an initial incubation at 94 °C for 5 min, followed by 45 cycles of amplification: 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min. A final elongation step was performed at 72 °C for 7 min and kept at 4 °C.

The PCR reaction products were resolved by electrophoresis in 1.5% agarose gels, stained with 500 nM of GelRed (Biotium, Hayward, CA, USA). If amplicons of the expected size were found, the PCR reaction was purified with DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA, USA), according to the manufacturer's protocol, and eluted in a final volume of 10 μL.

In positive samples, all the tissues available, including those in the initial tissue pool were subsequently tested separately, to map the HV infection. The amplified DNA was directly sequenced with the second round TGV and IYG primers by Sanger's method (STABVida, Portugal).

3.4. Phylogenetic analysis

The identity of the nucleotide sequences was confirmed by a nucleotide BLASTn suite (Altschul et al., 1990) of the Genbank database, with the default settings.

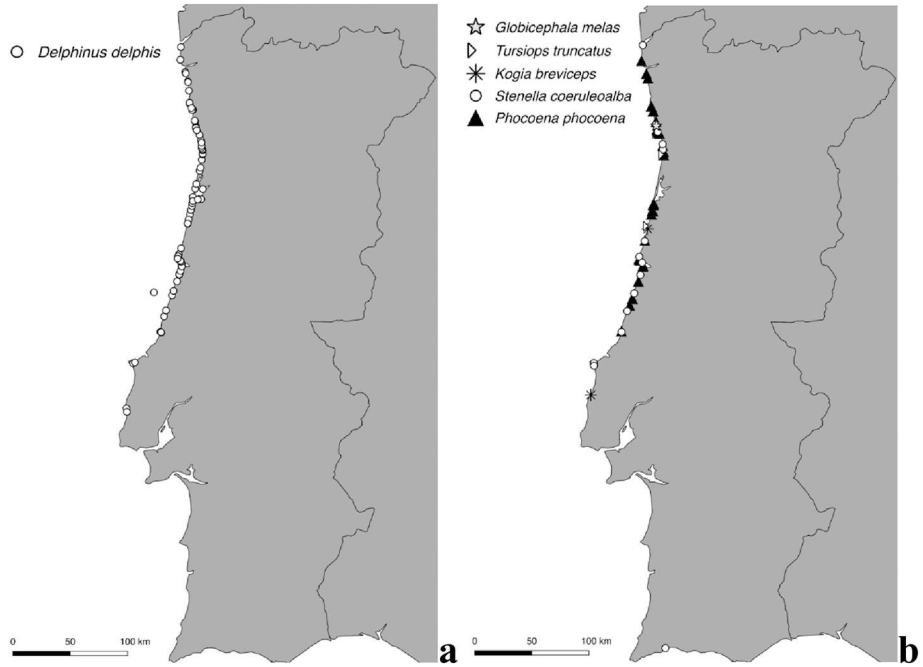

Fig. 1. Stranding sites for common dolphins (a) and other species (b) analyzed for herpesviral infection.

The sequences obtained in this study were imported to Unipro UGene (Okonechnikov et al., 2012), where the primer regions were excised. The resulting sequences were translated to the deduced amino acid sequences and all positions containing gaps and missing data were eliminated. Sequences were aligned using MUSCLE (Edgar, 2004), with the program's default settings. This alignment was imported into MEGA 7.0.21 (Kumar et al., 2016) and a phylogenetic tree was constructed using the Maximum Likelihood Method and the JTT matrix-model (Jones et al., 1992) with a discrete Gamma distribution to model the evolutionary rate differences among sites (5 categories (+G, parameter = 0.6180)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 9.2593% sites).

To construct the phylogenetic trees, sequences were retrieved from Genebank. Sequences belonging to alphaherpesvirus (30 sequences), gammaherpesvirus (19 sequences) and one sequence of betaherpesvirus. One sequence of an Iguanid Herpesvius was used as an outlier in one of the trees. Accession numbers for all the sequences used are specified in Table 2. Amino acid sequences that were 100% identical and did not add information to the phylogenetic tree were withdrawn from the alignment, and are identified within square brackets, in the phylogenetic tree.

3.5. Statistical analysis

The Fisher's exact test (http://vassarstats.net/) was performed to test for significant differences in the number of HV positive animals in relation to carcass conservation condition and according to cause of death, with a significance level set to p-value $\leq .05$.

3.6. Histology

For histology, tissue samples were processed as routine, embedded in paraffin and 4 µm sections were stained with hematoxylin and eosin (H&E) in an automated stainer for light microscopic analysis.

Immunohistochemical staining for detection of Toxoplasma gondii and Morbillivirus antigen was performed using the DAKO PT-link system and the Autostainer-EnVision (DAKO), according to already established routine procedures. For each sample positive and negative controls were used. The primary antibody against *Toxoplasma gondii* was a rabbit polyclonal antibody at a dilution of 1:4000 (courtesy of Dr. Bjerkas). The antibody against Cetacean morbillivirus (CeMV) was a monoclonal commercial antibody (VMRD) against the nucleoprotein of the Canine Distemper Virus that cross-reacts with CeMV, used at 1:50000 with an incubation step of 40 min. After the washing steps, the secondary antibody was incubated (DAKO Rabbit/Mouse EnVision Detection System (Dako Ref.: K5007) for 40 min at room temperature, with the dilution recommended by the manufacturer. Slides were incubated for 5 min in DAB-Chromogen-hydrogen peroxide (Dako k3468) and stained with Mayer hematoxylin.

4. Results

4.1. Herpesvirus nucleic acid detection and phylogenetic analysis

Out of the 179 tested animals, 14 (7 males; 7 females) were positive to herpesvirus by conventional PCR, corresponding to a percentage of 7.8% of positive animals (Table 3). Animals DD-105-2011 and DD-112-2011 were both positive for HV in the lung, the only available organ. Two different HV sequences were obtained from animal DD-317-2011. Two of the animals that were HV positive were also Dolphin Morbillivirus positive (SC-221-2012 and DD-302-2012) (Rocha De Medeiros Bento et al., 2016). Animal SC-189-2013 tested positive in the tissue pool, but not in the DNA extracted from the individual organs. Lung, kidney and liver were the organs most frequently tested. These were included in the pool of tissue samples that were used in the survey and were also tested individually in the HV positive pools. Lymph nodes and lesions were also tested in positive animals whenever they were available, and all the lymph nodes tested in the positive samples were positive, except for animal DD-230-2012 and DD-297-2011.

The number of positive samples was analyzed according to the cause of death: animals were divided into 2 categories; bycaught animals (11/128) and animals that died from disease or where cause of death was not determined (3/37). No association between cause of death and positive HV results were detected ($P = 1$).

The conservation score of the carcasses was also analyzed. Conservation scores range from 1 to 5, 1 being animals that were stranded alive, and 5 being the more decomposed carcasses. There was a significantly higher number of positive HV animals among fresh

Table 1
Stranded animals tested for herpesvirus: ID code, stranding date, species, carcass condition (1–5), sex and age class.

ID Code	Date of stranding	Species	Carcass condition	Sex	Age class
DD-100-2011	07/01/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-101-2011	09/01/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-103-2011	11/01/2011	*Delphinus delphis*	3	Female	ADULT
DD-105-2011	16/01/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-110-2011	27/01/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-111-2011	28/01/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-204-2011	28/01/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-112-2011	28/01/2011	*Delphinus delphis*	3	Female	ADULT
DD-113-2011	01/02/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-114-2011	05/02/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-115-2011	06/02/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-116-2011	07/02/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-117-2011	15/02/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-126-2011	07/03/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-125-2011	07/03/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-127-2011	16/03/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-128-2011	16/03/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-129-2011	26/03/2011	*Delphinus delphis*	2	Female	ADULT
DD-132-2011	30/03/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-133-2011	30/03/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-134-2011	01/04/2011	*Delphinus delphis*	3	Female	JUVENILE
dd-206-2011	02/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-137-2011	05/04/2011	*Delphinus delphis*	3	Female	ADULT
DD-141-2011	06/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-144-2011	06/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-156-2011	10/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-160-2011	11/04/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-158-2011	11/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-157-2011	12/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-161-2011	15/04/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-162-2011	18/04/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-166-2011	19/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-165-2011	20/04/2011	*Delphinus delphis*	4	Female	JUVENILE
DD-167-2011	21/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-169-2011	23/04/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-172-2011	29/04/2011	*Delphinus delphis*	4	Male	JUVENILE
DD-175-2011	02/05/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-176-2011	03/05/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-179-2011	04/05/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-177-2011	04/05/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-178-2011	04/05/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-183-2011	06/05/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-182-2011	06/05/2011	*Delphinus delphis*	4	Female	ADULT
DD-186-2011	08/05/2011	*Delphinus delphis*	4	Male	JUVENILE
DD-187-2011	09/05/2011	*Delphinus delphis*	4	Male	JUVENILE
DD-189-2011	10/05/2011	*Delphinus delphis*	3	Female	ADULT
DD-192-2011	10/05/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-193-2011	12/05/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-198-2011	19/05/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-250-2011	19/05/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-251-2011	19/05/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-199-2011	21/05/2011	*Delphinus delphis*	3	Female	JUVENILE
dd-216-2011	24/05/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-258-2011	27/05/2011	*Delphinus delphis*	3	Male	ADULT
dd-219-2011	09/06/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-261-2011	10/06/2011	*Delphinus delphis*	3	Female	ADULT
DD-269-2011	12/07/2011	*Delphinus delphis*	3	Female	JUVENILE
dd-223-2011	14/07/2011	*Delphinus delphis*	2	Male	ADULT
DD-287-2011	03/10/2011	*Delphinus delphis*	3	Male	ADULT
dd-224-2011	07/10/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-288-2011	13/10/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-290-2011	30/10/2011	*Delphinus delphis*	3	Male	ADULT
DD-291-2011	09/11/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-293-2011	23/11/2011	*Delphinus delphis*	3	Male	ADULT
DD-294-2011	23/11/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-297-2011	09/12/2011	*Delphinus delphis*	2	Female	JUVENILE
DD-304-2011	10/12/2011	*Delphinus delphis*	2	Female	CALF
DD-299-2011	10/12/2011	*Delphinus delphis*	3	Female	ADULT
DD-302-2011	12/12/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-307-2011	14/12/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-306-2011	14/12/2011	*Delphinus delphis*	2	Male	JUVENILE
DD-308-2011	14/12/2011	*Delphinus delphis*	3	Male	ADULT
dd-310-2011	15/12/2011	*Delphinus delphis*	3	Not determined	not determined
DD-309-2011	15/12/2011	*Delphinus delphis*	4	Female	JUVENILE

(*continued on next page*)

Table 1 (*continued*)

ID Code	Date of stranding	Species	Carcass condition	Sex	Age class
DD-317-2011	16/12/2011	*Delphinus delphis*	1	Female	JUVENILE
DD-316-2011	17/12/2011	*Delphinus delphis*	3	Male	ADULT
DD-318-2011	21/12/2011	*Delphinus delphis*	2	Female	ADULT
DD-320-2011	27/12/2011	*Delphinus delphis*	3	Male	JUVENILE
DD-319-2011	27/12/2011	*Delphinus delphis*	3	Female	JUVENILE
DD-321-2011	28/12/2011	*Delphinus delphis*	3	Female	ADULT
DD-159-2012	07/02/2012	*Delphinus delphis*	3	Female	ADULT
DD-160-2012	09/02/2012	*Delphinus delphis*	3	Female	ADULT
DD-163-2012	16/02/2012	*Delphinus delphis*	3	Female	ADULT
DD-164-2012	20/02/2012	*Delphinus delphis*	4	Male	JUVENILE
DD-165-2012	21/02/2012	*Delphinus delphis*	3	Female	ADULT
DD-167-2012	23/02/2012	*Delphinus delphis*	2	Female	JUVENILE
DD-168-2012	23/02/2012	*Delphinus delphis*	2	Female	ADULT
DD-169-2012	25/02/2012	*Delphinus delphis*	3	Female	JUVENILE
DD-176-2012	02/03/2012	*Delphinus delphis*	3	Female	ADULT
DD-178-2012	03/03/2012	*Delphinus delphis*	3	Female	JUVENILE
DD-179-2012	03/03/2012	*Delphinus delphis*	3	Female	ADULT
DD-186-2012	11/03/2012	*Delphinus delphis*	3	Female	JUVENILE
DD-199-2012	16/03/2012	*Delphinus delphis*	3	Male	ADULT
DD-200-2012	16/03/2012	*Delphinus delphis*	3	Male	ADULT
DD-206-2012	22/03/2012	*Delphinus delphis*	2	Male	JUVENILE
DD-212-2012	22/03/2012	*Delphinus delphis*	2	Female	ADULT
DD-213-2012	24/03/2012	*Delphinus delphis*	2	Male	JUVENILE
DD-217-2012	31/03/2012	*Delphinus delphis*	2	Female	JUVENILE
DD-219-2012	31/03/2012	*Delphinus delphis*	3	Female	JUVENILE
DD-224-2012	04/04/2012	*Delphinus delphis*	3	Female	JUVENILE
DD-230-2012	07/04/2012	*Delphinus delphis*	2	Male	JUVENILE
DD-231-2012	09/04/2012	*Delphinus delphis*	3	Female	ADULT
DD-241-2012	23/04/2012	*Delphinus delphis*	3	Male	JUVENILE
DD-253-2012	01/05/2012	*Delphinus delphis*	2	Male	JUVENILE
DD-277-2012	10/09/2012	*Delphinus delphis*	2	Male	JUVENILE
DD-278-2012	14/09/2012	*Delphinus delphis*	2	Female	JUVENILE
DD-280-2012	27/09/2012	*Delphinus delphis*	3	Female	JUVENILE
DD-135-2012	01/11/2012	*Delphinus delphis*	3	Female	ADULT
DD-134-2012	01/11/2012	*Delphinus delphis*	3	Male	ADULT
DD-296-2012	12/11/2012	*Delphinus delphis*	2	Male	JUVENILE
DD-297-2012	12/11/2012	*Delphinus delphis*	2	Female	JUVENILE
DD-302-2012	04/12/2012	*Delphinus delphis*	1	Female	JUVENILE
dd-150-2013	02/01/2013	*Delphinus delphis*	2	Female	JUVENILE
DD-151-2013	02/01/2013	*Delphinus delphis*	2	Female	ADULT
DD-155-2013	13/01/2013	*Delphinus delphis*	2	Female	JUVENILE
DD-156-2013	14/01/2013	*Delphinus delphis*	3	Male	JUVENILE
DD-172-2013	11/03/2013	*Delphinus delphis*	2	Female	ADULT
DD-188-2013	22/05/2013	*Delphinus delphis*	3	Male	JUVENILE
DD-191-2013	02/06/2013	*Delphinus delphis*	1	Female	JUVENILE
DD-203-2013	19/07/2013	*Delphinus delphis*	2	Male	CALF
DD-205-2013	19/07/2013	*Delphinus delphis*	2	Female	JUVENILE
DD-212-2013	19/07/2013	*Delphinus delphis*	2	Female	ADULT
DD-213-2013	19/07/2013	*Delphinus delphis*	2	Female	ADULT
DD-209-2013	19/07/2013	*Delphinus delphis*	2	Male	ADULT
DD-211-2013	19/07/2013	*Delphinus delphis*	2	Male	ADULT
dd-213-2014	25/03/2014	*Delphinus delphis*	3	Female	JUVENILE
GM-272-2012	29/07/2012	*Globicephala melas*	1	Male	JUVENILE
KB-271-2012	21/07/2012	*Kogia breviceps*	1	Male	JUVENILE
kb-266-2014	17/05/2014	*Kogia breviceps*	1	Male	ADULT
PP-102-2011	11/01/2011	*Phocoena phocoena*	3	Male	ADULT
PP-135-2011	01/04/2011	*Phocoena phocoena*	3	Male	ADULT
PP-184-2011	08/05/2011	*Phocoena phocoena*	4	Female	ADULT
PP-191-2011	10/05/2011	*Phocoena phocoena*	3	Female	CALF
PP-252-2011	19/05/2011	*Phocoena phocoena*	4	Female	JUVENILE
PP-253-2011	20/05/2011	*Phocoena phocoena*	2	Female	JUVENILE
PP-255-2011	21/05/2011	*Phocoena phocoena*	2	Female	YEARLING
PP-223-2011	18/06/2011	*Phocoena phocoena*	4	Not determined	JUVENILE
PP-270-2011	11/07/2011	*Phocoena phocoena*	2	Female	YEARLING
PP-273-2011	31/07/2011	*Phocoena phocoena*	2	Female	CALF
PP-282-2011	24/08/2011	*Phocoena phocoena*	1	Female	ADULT
PP-284-2011	27/08/2011	*Phocoena phocoena*	3	Female	ADULT
PP-285-2011	28/08/2011	*Phocoena phocoena*	3	Female	JUVENILE
PP-286-2011	02/10/2011	*Phocoena phocoena*	3	Female	ADULT
PP-313-2011	14/12/2011	*Phocoena phocoena*	3	Male	JUVENILE
PP-311-2011	15/12/2011	*Phocoena phocoena*	3	Female	JUVENILE
PP-322-2011	29/12/2011	*Phocoena phocoena*	3	Male	YEARLING
PP-175-2012	28/02/2012	*Phocoena phocoena*	3	Male	JUVENILE
PP-211-2012	18/04/2012	*Phocoena phocoena*	3	Male	JUVENILE
PP-266-2012	08/06/2012	*Phocoena phocoena*	3	Male	YEARLING

(*continued on next page*)

Table 1 (*continued*)

ID Code	Date of stranding	Species	Carcass condition	Sex	Age class
PP-267-2012	21/06/2012	*Phocoena phocoena*	2	Female	JUVENILE
PP-268-2012	30/06/2012	*Phocoena phocoena*	3	Male	ADULT
PP-275-2012	20/08/2012	*Phocoena phocoena*	4	Female	ADULT
PP-276-2012	24/08/2012	*Phocoena phocoena*	2	Female	ADULT
PP-184-2013	12/05/2013	*Phocoena phocoena*	3	Female	JUVENILE
PP-196-2013	19/06/2013	*Phocoena phocoena*	3	Male	JUVENILE
pp-236-2013	21/08/2013	*Phocoena phocoena*	2	Male	ADULT
pp-251-2013	16/10/2013	*Phocoena phocoena*	2	Female	JUVENILE
pp-271-2013	30/12/2013	*Phocoena phocoena*	2	Female	JUVENILE
pp-205-2014	22/03/2014	*Phocoena phocoena*	3	Female	JUVENILE
pp-227-2014	08/04/2014	*Phocoena phocoena*	3	Male	JUVENILE
SC-119-2011	17/02/2011	*Stenella coeruleoalba*	3	Female	JUVENILE
SC-159-2011	11/04/2011	*Stenella coeruleoalba*	3	Male	JUVENILE
SC-257-2011	22/05/2011	*Stenella coeruleoalba*	1	Male	JUVENILE
SC-260-2011	07/06/2011	*Stenella coeruleoalba*	2	Male	JUVENILE
SC-298-2011	10/12/2011	*Stenella coeruleoalba*	1	Female	JUVENILE
SC-209-2012	19/03/2012	*Stenella coeruleoalba*	3	Female	JUVENILE
SC-210-2012	22/03/2012	*Stenella coeruleoalba*	2	Female	ADULT
SC-251-2012	24/04/2012	*Stenella coeruleoalba*	2	Unassigned	CALF
SC-249-2012	24/04/2012	*Stenella coeruleoalba*	3	Female	ADULT
SC-221-2012	03/08/2012	*Stenella coeruleoalba*	1	Male	ADULT
SC-274-2012	12/08/2012	*Stenella coeruleoalba*	3	Female	CALF
SC-182-2013	30/04/2013	*Stenella coeruleoalba*	1	Female	YEARLING
SC-189-2013	31/05/2013	*Stenella coeruleoalba*	1	Female	JUVENILE
SC-200-2013	02/07/2013	*Stenella coeruleoalba*	2	Male	JUVENILE
sc-188-2014	26/02/2014	*Stenella coeruleoalba*	2	Male	JUVENILE
sc-193-2014	03/03/2014	*Stenella coeruleoalba*	3	Male	JUVENILE
SC-12-2014	08-03-2014	*Stenella coeruleoalba*	1	Female	JUVENILE
TT-222-2011	19/06/2011	*Tursiops truncatus*	2	Female	ADULT
TT-233-2012	12/04/2012	*Tursiops truncatus*	2	Male	ADULT

animals (conservation scores 1 and 2; $n = 70$) in comparison to the group of samples from the decomposed animals (conservation score 3 and 4; $n = 109$) ($P = 0{,}024$). When considering samples from fresh carcasses only, the percentage of HV positive animals increases up to 14.3% (10/70).

Sequences obtained in this study were uploaded to an online database (Genbank) and are available online with sequential accession numbers from MG437203 to MG437217.

4.2. Phylogenetic analysis

To clarify the taxonomic assignment of the HV sequences a tree was constructed (Fig. 1B) with sequences from the *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpervirinae* subfamilies. For the *Alphaherpesvirinae* subfamily, four different genera were chosen: *Simplexvirus* (Herpes simplex virus type 1; Eidolon helvum simplexvirus 1); *Varicellovirus* (Equid herpesvirus 1; Suid herpesvirus 1); *Mardivirus* (Collumbid herpesvirus 1) and *Iltovirus* (Passerid Herpesvirus 1). For the subfamily *Betaherpesvirinae,* one sequence of Human herpesvirus 6A was included, and to represent the *Gammaherpervirinae* subfamily, a sequence of Human herpesvirus 4. Within the Alphaherpesvirus the two *Simplexvirus* sequences were grouped together (BS 91%) as well as the two *Varicellovirus* sequences, despite the lower bootstrap value of this cluster (BS 44%). The *Iltovirus* and *Mardivirus* sequences were isolated in separate branches. The Portuguese HV sequences belonging to the *Alphaherpesvirinae* subfamily were distributed in three separate branches, without clustering in either of the HV genera. Sequences DD317/2011b and SC221/2012 clustered together (α1) with a high bootstrap value (BS 100). Sequences DD317/2011a; PP273/2011; DD302/2012; DD206/2012; DD297/2011 clustered in the same branch (α2) although supported by a lower bootstrap value (49%). The α3 branch included sequences DD141/2011, DD132/2011, PP271/2013 and DD112/2011 (BS 89%). In the *Gammaherpesvirinae* branch (BS 86%), the inclusion of the HHV4 (AJ507799) sequences did not resolve the four Portuguese HV sequences (SC189/2013; DD183/2011; DD230/2012 and DD105/2011) positioning. No Portuguese HV sequences clustered within the *Betaherpesvirinae* branch.

The evolutionary history was inferred using the ML method by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT + G + I model, and then selecting the topology with superior log likelihood value (Jones et al., 1992). Trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

A phylogenetic analysis was performed including the available HV sequences detected in marine mammals and previously characterized. In this tree, (Fig. 2) the Portuguese HVs sequences obtained in the present study form two distinct branches supported by high bootstrap (BS) values, corresponding to the subfamilies *Alphaherpesvirinae* and *Gammaherpesvirinae*. In one branch with 78% BS, sequences previously identified as alphaherpesvirus and the Portuguese samples: DD-112/2011, DD-132/2011, DD-141/2011, PP-273/2011, DD-297/2011, DD-317/2011a, DD-317/2011b, DD-206/2012, SC-221/2012, DD-302/2012 and PP-271/2013, clustered together. In the other branch with a 100% BS, all sequences previously identified as gammaherpesvirus clustered with the Portuguese samples: PP-105/2011, DD-112/2011, DD-183/2011, DD-230/2012 and SC-189/2013. Gammaherpesvirus represented in the tree belong to two Blainville's beaked whales; one porpoise; a Risso's dolphin; two minke whales; ten bottlenose dolphins; a striped dolphin and a dwarf sperm whale.

The *Alphaherpesvirinae* subfamily sequences were further divided into three different monophyletic branches; α-1, α-2 and α-3; each supported by high BS values (86, 77 and 80% respectively). Each group included HV sequences amplified from animals belonging to different species. The α-1 group included sequences from a Cuvier's beaked whale, an orca, and bottlenose and striped dolphins, together with the Portuguese HV sequences from a common dolphin (DD-371/2011b) and a striped dolphin (SC-221/2012). The α-2 group included

Table 2
Accession numbers, identification and country of origin of the sequences used in the phylogenetic trees.

Subfamily	Accession number	Organism	Host	Country
Alphaherpesvirus	GQ888674	Unidentified herpesvirus	*Stenella coeruleoalba*	Spain
	AY608707	*Tursiops truncatus* alphaherpesvirus 1	tursiops truncatus	Germany
	GQ429151	Delphinid herpesvirus 9	*Orcinus orca*	USA
	GU066291	Herpesvirus whale	*Ziphius cavirostris*	Spain (canary islands)
	GU068981	Unidentified herpesvirus	Stenella coeruleoalba	Spain
	HQ214675	Unidentified herpesvirus	Stenella coeruleoalba	Spain
	AY949832	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
	AF196646	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
	GQ888671	Unidentified herpesvirus	stenella coeruleoalba	Spain
	JN863234	*Mesoplodon densirostris* herpesvirus	Mesoplodon densirostris	Spain (canary islands)
	KP995686	*Balaenoptera physalus* alphaherpesvirus	Balaenoptera physalus	Spain
	GQ888669	Unidentified herpesvirus	stenella coeruleoalba	Spain
	AB510474	Melon-headed whale alphaherpesvirus	*Peponocephala electra*	Japan
	GQ888673	Unidentified herpesvirus	stenella coeruleoalba	Spain
	AF245443	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
	DQ295064	Tursiops truncatus alphaherpesvirus 3	tursiops truncatus	Germany
	GQ429150	Delphinid herpesvirus 8	tursiops truncatus	USA
	AB510473	False killer whale alphaherpesvirus	*Pseudorca crassidens*	Japan
	GQ888675	unidentified herpesvirus	stenella coeruleoalba	Spain
	GQ888670	unidentified herpesvirus	stenella coeruleoalba	Spain
	DQ295063	Tursiops truncatus alphaherpesvirus 2	tursiops truncatus	Germany
	AY757301	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
	JQ692312	Equid alphaherpesvirus 1	*Ursus maritimus*	Germany
	KP279684	Suid alphaherpesvirus 1		China
	M10792	Human alphaherpesvirus 1		
	FJ040890	*Eidolon helvum* simplexvirus 1	Eidolon helvum	Cameroon
	KJ995972	Columbid alphaherpesvirus 1	pigeon	China
	AF520812	Passerid herpesvirus 1	Gouldian finch	
Gammaherpesvirus	AY803337	Blainville's beaked whale gammaherpesvirus	Mesoplodon densirostris	USA
	AY949828	Blainville's beaked whale gammaherpesvirus	Mesoplodon densirostris	USA
	KT591613	Phocoenid herpesvirus 1	*Phocoena phocoena*	Netherlands
	DQ288666	Risso's dolphin gammaherpesvirus	*Grampus griseus*	USA
	GQ888672	unidentified herpesvirus	stenella coeruleoalba	Spain
	AY949830	Dwarf sperm whale gammaherpesvirus	*Kogia simus*	USA
	KP995688	*Balaenoptera acutorostrata* gammaherpesvirus 2	Balaenoptera acutorostrata	Spain
	AY949831	Bottlenose dolphin gammaherpesvirus	tursiops truncatus	USA
	AY952777	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
	AY952779	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
	GQ258355	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	GQ258356	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	GQ258353	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	GQ258354	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	AY952776	Atlantic bottlenose dolphin gammaherpesvirus	tursiops truncatus	USA
	DQ288667	Atlantic bottlenose dolphin gammaherpesvirus	tursiops truncatus	USA
	KP995687	Balaenoptera acutorostrata gammaherpesvirus 1	Balaenoptera acutorostrata	Spain
	KC142153	Bottlenose dolphin herpesvirus 201MG_R	tursiops truncatus	Italy
	AJ507799	Human gammaherpesvirus 4		
Betaherpesvirus	NC_001664	Human betaherpesvirus 6A	*Homo sapiens*	Uganda

sequences from a Blainville's beaked whale, a fin whale, a melon headed whale, bottlenose, striped dolphins and the Portuguese sequences of a porpoise (PP-273/2011) and common dolphins: DD-317/2011a; DD-302/2012; DD-206/2012; DD-297/2011. In the α-3 group, sequences from a false killer whale, bottlenose and striped dolphins grouped with the Portuguese common dolphin sequences DD-112/2011, DD-141/2011 and DD-132/2011 and a porpoise sequence (PP-271/2013).

The evolutionary history was inferred using the ML method by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT + G + I model, and then selecting the topology with higher log likelihood value (Jones et al., 1992). Trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Portuguese sequences are marked with an asterisk.

4.3. Gross and microscopical findings

Histopathological examination was performed in seven positive animals: SC-221/2012, DD-302/2012, DD-317/2011, DD-112/2011, DD-230/2012, SC-189/2013, and PP-271/2013. Stranding condition, relevant findings and cause of death are summarized in Table 4.

Animal SC-221/2012 was a live stranding that died during rescue efforts at the beach. The animal presented ectoparasites and ulcerations in the mouth and a poor body condition. It tested positive both for CeMV (Rocha De Medeiros Bento et al., 2016) and HV nucleic acid, although it was negative in the immunohistochemistry (IHQ) for CeMV in the lung, diaphragmatic lymph node, spleen and central nervous system. In the histological findings, the animal showed purulent catarrhal bronchopneumonia and lymphoid depletion with necrosis of the lymphoid organs. Giant syncytial cells with basophilic intranuclear inclusion bodies were detected in the lymph nodes and lungs. Furthermore, this animal presented a light focal mononuclear encephalitis, a mononuclear interstitial (periportal) hepatitis and a parasitic gastritis in

Table 3
Tested and positive samples for herpesvirus antigen detected by conventional nested-PCR.

	Pool	Liver	Kidney	Lung	Brain	Spleen	Regional lymph nodes			Genital lesion
							Pulmonary	Mesenteric	Pre-escapular	
DD-105-2011				X						
DD-112-2011				X						
DD-132-2011	X	X	X							
DD-141-2011	X		X							
DD-183-2011	X	X	X							
PP-273-2011	X	X	X	X			X			
DD-297-2011	X	X								
DD-317-2011	X		X	X				X	X	
DD-206-2012	X		X	X			X			
SC-221-2012	X		X	X			X			
DD-230-2012	X	X								X
DD-302-2012	X	X	X	X	X		X			
SC-189-2013	X									
PP-271-2013	X				X					

Grey cells indicate the tested samples; positive samples are marked with an X.

Grey cells indicate the tested samples; positive samples are marked with an X.

the second and third stomachs. Lesions in the lymphoid organs and central nervous system as well as the morphology of the observed inclusion bodies were consistent with herpesvirus infection.

Animal DD-302/2012, similarly to animal SC-221/2012, was also a live stranding and was positive for both CeMV and HV nucleic acids by molecular detection, and negative in the IHQ for CeMV in the lung, diaphragmatic lymph node, thyroids and central nervous system. The histological findings included a generalized lymphoid depletion with amyloid deposition in the lymph nodes; additionally, an active toxoplasmosis was detected, with lesions in the liver and adrenal glands, including a necrotizing lymphoplasmacytic hepatitis and a necrotizing adrenalitis. No *Toxoplasma gondii* infection was detected in the CNS or lymph nodes.

Animal DD-317/2011 stranded alive, died 48 h after rescue and transport to the rehabilitation center. A subacute bronchointersticial pneumonia was identified as well as apoptotic lesions in the liver, most likely due to hypoxia or toxicity. Pneumonia was the most relevant lesion, most probably due to a primary viral infection followed by a subsequent bacterial infection. The liver lesions were mild to moderate and could have been a result of the stranding process.

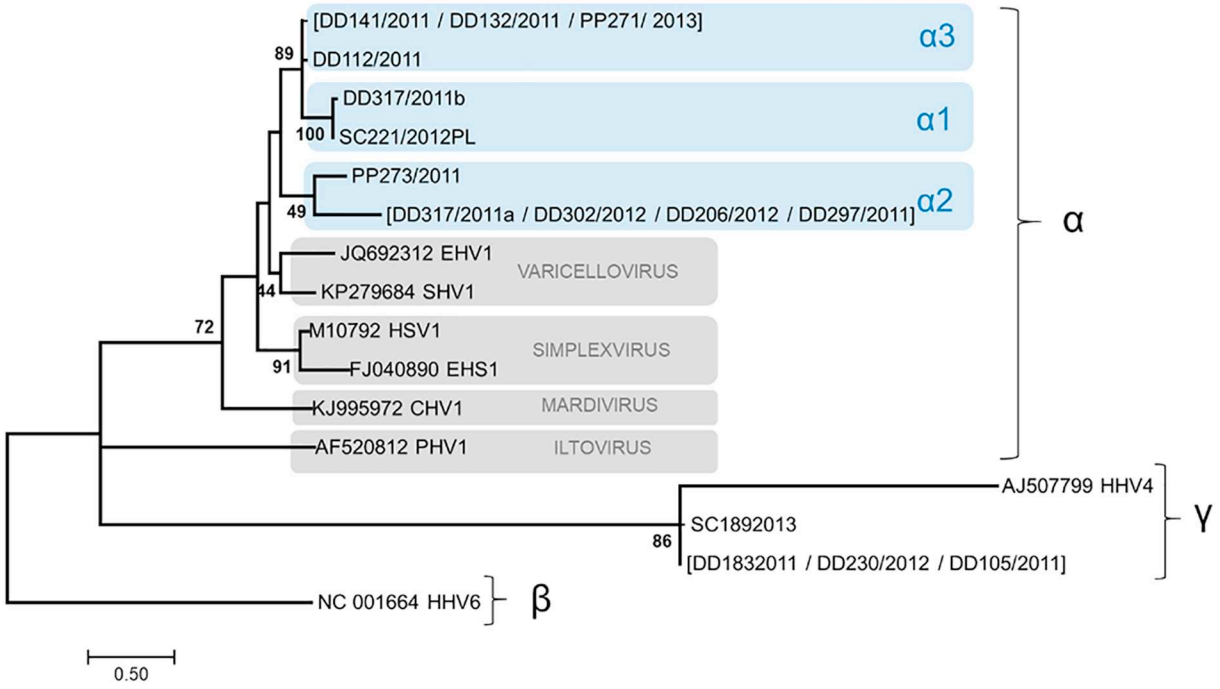

Fig. 2. Molecular Phylogenetic analysis of Portuguese sequences, subfamily alpha gamma and betaherpesvirus, with alphaherpesvirus genera represented.

Table 4
Stranding condition, body condition, most significant findings (gross and microscopic), virology results and cause of death for animals in which histopathology was performed.

ID code	Stranding condition	Body condition	Gross and microscopic findings	Virology	Cause of death
SC-221/2012	Live Stranding	Poor	Ectoparasites and ulcerations in the mouth, pneumonia, encephalitis, hepatitis and lymphoid depletion with necrosis: consistent with HV infection	+ Hv; + CeMV	Pneumonia/encephalitis
DD-302/2012	Live Stranding	poor	Active toxoplamosis with hepatitis and adrenalitis. Generalized lymphoid depletion.	+ Hv; + CeMV	toxoplasmosis
DD-317/2011	Live Stranding	Poor	Bronchointersticial pneumonia, most likely of viral origin	+HV	Pneumonia
DD-112/2011	Found dead	NR	No lesions reported; aspiration of foreign material.	+HV	not determined
DD-230/2012	Live Stranding	Good	Parasitic gastritis with ulcers in the first stomach and a moderate to heavy load of nematodes	+HV	Bycatch
SC-189/2013	Live Stranding	poor	Heavy load of ectoparasites and extensive pox-like lesions in the skin	+HV	euthanasia
PP-271/2013	Found dead	Good	Severe necrotizing hemorrhagic gastritis in the second stomach	+HV	Bycatch/ gastritis

Animal DD-112/2011 did not show clear signs of disease and the only relevant observation in histology was the presence of foreign material in the lungs (muscle fibers with nucleated erythrocytes), probably due to agonal aspiration.

Animal DD-230/2012 stranded alive twice before being found dead at the beach. This animal presented a parasitic gastritis with ulcers in the first stomach and a moderate to heavy load of nematodes. It had a genital lesion that tested positive to herpesvirus and a lymphoplasmacytic urethritis was identified in the histological exam, with possible viral inclusion bodies in the ureteral epithelium. These lesions were unlikely to have caused death and this animal was in good body condition.

Animal SC-189/2013 was a live stranding that was euthanized. This animal had a heavy load of ectoparasites and extensive pox-like lesions in the skin and a mild purulent catarrhal bronchopneumonia. The thymus and lymph nodes had intra-nuclear acidophilic inclusion bodies in lymphoid cells and syncytial giant cells. It had a superficial necrotizing dermatitis with thrombi, perivascular infiltrates and corneal micro-pustules. In the areas with lesions, keratinocytes seem to have cytoplasmic inclusions; highly suggestive of a viral infection. Although this animal tested positive for HV nucleic acid in the tissue pool, it was not possible to map the infection.

Animal PP-271/2013 had signs of bycatch. Its histology presented a severe necrotizing hemorrhagic gastritis in the second stomach. Etiology was not determined, but it was possible that these lesions could be due to *Clostridium* spp., which could have caused a fatal toxemia.

Bycatch was the cause of death determined at necropsy for several animals: DD-183/2011, DD-230/2012 and DD-205/2011, all positive to gammaherpesvirus; and PP-273/2911, DD-206/2012, DD-297/2011, DD-141/2011 and DD-132/2011, positive to alphaherpesvirus.

5. Discussion

A systematic approach is necessary to implement health monitoring in wild marine mammals' populations. Routine collection of tissue samples from stranded animals contributes to the establishment of a tissue bank, providing available samples for health surveillance. Systematic surveys play an important role in epidemiological surveillance and allow the detection of emerging and resurgence of infectious diseases (Delwart, 2012; Gilbert et al., 2011). In unusual mortality events (UME) or disease outbreaks, such surveys are even more critical for determining the cause of the mortality. Routine evaluations with established baselines and established laboratory working relationships are essential for such investigations.

In this work we surveyed 179 cetaceans stranded along the Portuguese coastline and found a total percentage of positive animals of 7.8% (14/179). Since carcass condition score was found to be related with HV DNA polymerase detection, while the percentage of HV positive animals rises to 14.3% (10/70) when considering only samples from fresh carcasses. This percentage is closer to values reported in similar studies using conventional PCR detection systems: 25.8% in chimpanzees (Seimon et al., 2015), 27% in rodents (Ehlers et al., 2007) and 26% in humans (Minjolle et al., 1999), although still inferior. Considering that, although the target viability is not necessary for PCR assays, maintenance of the biological matrix under optimum conditions for virus detection will enhance pathogen detection by these techniques. Therefore, samples should be correctly collected and maintained in order to improve antigen detection by molecular and serological assays (MacLachlan and Dubovi, 2011b; Storch and Wang, 2013). Considering our results, we recommend that all available samples should be tested to detect the highest number of sequences possible. However, if resources are scarce, we would recommend testing only fresh samples, especially when estimating disease prevalence. Otherwise, the results can be considerably underestimated.

Although herpesvirus in cetaceans have been documented since

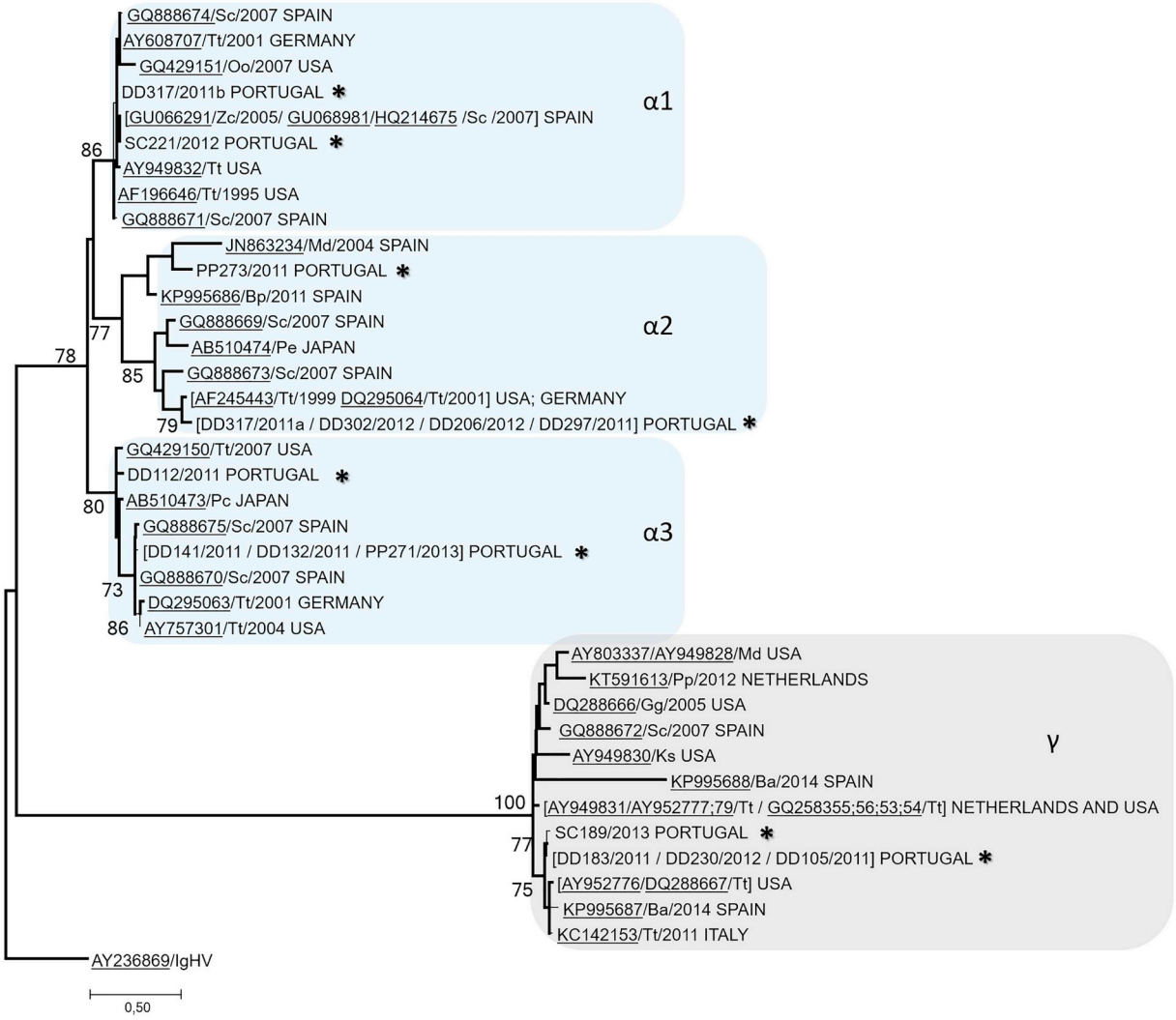

Fig. 3. Molecular Phylogenetic analysis of Portuguese sequences and sequences previously detected in marine mammals.

1988 (Martineau et al., 1988), the available information is still scarce (Lecis et al., 2014). The pan-herpesvirus primers used in this work allowed the detection of herpesvirus in species with no previous reports of herpesvirus infection, such as the pacific walrus, fin whale, minke whale or even box turtles (Melero et al., 2015, Melero et al., 2014; Sim et al., 2015). Our work is the first time herpesvirus infection was reported in common dolphins, with both alpha and gammaherpesvirus detected. The sequences obtained in this species do not seem to be species-specific, similarly to what happens in the other species represented in the tree.

In cetaceans, there are still very few sequences available and more studies are needed to assess the diversity of herpesvirus in these species. Furthermore, few published studies focused on the prevalence of infection in cetaceans (Elk et al., 2016; Sierra et al., 2014) and this is one of the few studies that systematically tested stranded cetaceans for herpesvirus antigen by analyzing samples from 2011 to 2014. The development of specific primers for the amplification of cetacean herpesvirus, could provide a more robust molecular assay, allowing a more rapid and efficient detection of positive samples.

Concerning the phylogeny of the reported Portuguese HV sequences, and although the short Dpol sequence available allows only for the subfamily identification (Maness et al., 2011), the phylogenetic tree generated from the obtained and previously reported sequences displayed three distinct clusters within the Portuguese alpha-herpesvirus, circulating in Atlantic waters off the coast of Portugal. None of these Portuguese sequences clustered with any of the previously identified genera (Fig. 3b).

The Dpol region is highly conserved in all the HVs, making it the gene of choice for examining phylogenetic relationships, since homologous sites give a more reliable alignment (VanDevanter et al., 1996). However, it does not allow a more precise phylogenetic analysis (Ehlers et al., 2008), only achieved with the concatenated alignments of up to 8 core genes in mammalian HVs (McGeoch et al., 2006),.

In one of the positive animals (DD-317/2011), two different sequences of herpesvirus were co-amplified. Although they were both included within the Alphaherpesvirinae cluster, one sequence grouped within the α-1 group (DD-317/2011b), while the other sequence grouped in the α-2 group (DD-317/2011a), sharing 59% of the nucleotide sequence identity. Co-infection with different viral strains was already identified. Bellière et al. (2010) reported the detection of HV sequences from more than one strain, in three of five dolphins (3/5; 60%) infected with both HV and CeMV. Later, Sierra et al. (2014) reported four new α-herpesvirus sequences, two of them detected in the same animal. Also, three different sequences of α-herpesvirus were detected in the skin, spleen and blood of a single bottlenose dolphin stranded in Germany (Smolarek Benson et al., 2006).

In the present study, animals SC-221/2012 and DD-302/2012 were both co-infected with herpesvirus and morbillivirus and animal DD-302/2012 was also co-infected with *Toxoplasma*. The histological evaluation performed in 7 of the 14 positive animals showed that, although

in some cases no lesions attributable to HV infection were found (e.g. animal DD-112/2011), in others there were clear signs of this viral infection. Animal SC-189-2013 had signs of a systemic viral infection, and animal DD-230/2012 showed localized lesions in the ureteral epithelium and an associated genital lesion, positive to HV. In both cases, the amplified HV sequences clustered with gammaherpesvirus sequences (Fig. 1A and B). *Gammaherpesvirinae* can display very different pathophysiology's and so far in cetaceans they have only been found associated to skin and genital lesions (van Beurden et al., 2015; Lecis et al., 2014; Smolarek Benson et al., 2006), which concurs with the macroscopic lesions observed in animal DD-230/2012. However, in other animal species gamma-herpesvirus have been found associated to fatal systemic infections, such as the malignant catarrhal fever affecting ruminants (Russell et al., n.d.). In animal SC-189/2013 with signs of a systemic viral infection, we cannot exclude the presence of a different HV, justifying the differences between the histological evaluation, especially since we could not map the infection in the different organs.

In a previously published paper by Sierra et al., 2014, it was suggested that there could be a pathogenic branch of alpha-herpesviruses in cetaceans that could be responsible for fatal cases worldwide. In our study, animals SC-221/2012 and DD-317/2011 corroborate this hypothesis, with signs of viral disease severe enough to cause death.

6. Conclusion

Molecular detection of viral antigen in animals with no signs of disease is consistent with the epidemiology of herpesviruses that have the ability to remain latent for long periods of time and also reinforces the fact that these viruses most likely evolved with their hosts and are well adapted to them. Despite this fact, these viruses are also capable of causing severe disease, especially when associated to other pathogenic agents such as morbilliviruses and toxoplasmosis.

Our results suggest that herpesvirus infection plays an important role in cetacean morbidity. We detected herpesvirus in common dolphins, porpoises and striped dolphins. To our knowledge, this work is the first report of herpesvirus infection in common dolphins (*Delphinus delphis)* in the Atlantic Ocean; providing 11 new sequences of herpesvirus in this species. Although animals from different species were analyzed and compared to existing sequences, it is important to highlight that sequences from different species cluster together in the phylogenetic tree, pointing to the idea that these viruses are not species-specific.

The virus was detected in animals with severe systemic disease, particularly when associated with morbillivirus infection. Also, animals with mild localized lesion caused by herpesvirus infection were detected. Animals that showed moderate or severe systemic disease had alphaherpesvirus detected, while gammaherpesvirus was mostly found in mild or localized lesions.

This study allowed the detection and amplification of 15 new sequences of the conserved DNA polymerase of herpesvirus affecting cetaceans.

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