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DIANA VIEIRA JORGE

ORIENTADORA:
DOUTORA ANA ISABEL SIMÕES PEREIRA DUARTE
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Anexo 3 – DECLARAÇÃO RELATIVA ÀS CONDIÇÕES DE REPRODUÇÃO DA TESE OU DISSERTAÇÃO

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Resumo

Rastreio virológico em cetáceos arrojados do norte de Portugal – considerações sobre poxvírus e o coronavírus cetáceo

Com uma costa marítima extensa, as águas portuguesas albergam diferentes espécies animais, residentes e migratórias. Registam também um elevado número de arrojamentos anuais (aproximadamente 236 anuais), permitindo a recolha padronizada de amostras biológicas e a criação de um Banco de Tecidos pela Rede de Arrojamentos e ICNF.

O Poxvírus dos cetáceos é responsável pelo aparecimento de lesões cutâneas muito características e já amplamente estudadas (lesões *pinhole* ou em forma de anel) e pela *tattoo skin disease* (TSD). Presentemente são reconhecidos 6 grupos filogenéticos distintos (CePV1-6). Embora este vírus não esteja relacionado com altas taxas de mortalidade, pode ser responsável pela mortalidade de neonatos e juvenis sem imunidade materna e de pequenas populações isoladas.

Um total de 47 amostras de lesões de pele, colhidos entre os anos de 2011 e 2015, e pertencentes a 4 espécies diferentes de golfinhos (*Delphinus delphis, Tursiops truncates, Phocoena phocoena* e *Stenella coeruleoalba*), foram analisadas por PCR convencional, utilizando um conjunto de oligonucleotideos degenerados. As amostras positivas (n=28, 58,33%) foram posteriormente testadas para o gene da DNA polimerase, onde 19 (40,4%) testaram positivo. Os produtos foram posteriormente sequenciados, e as sequências amino acídicas utilizadas na construção de uma árvore filogenética. As sequências dos poxvírus nacionais mostraram-se geneticamente mais próximos dos grupos CePV-1, 5, 3 e 6. Foi também identificado um novo agrupamento das amostras sequenciadas, sugerindo a existência de um sétimo grupo (CePV-7).

Contrariamente ao Poxvirus, o coronavírus cetáceo foi apenas recentemente identificado e incluído no género Gammacoronavirus. Desde a sua identificação numa baleia beluga em 2008, este vírus foi detetado anos mais tarde em Hong Kong e nos Estados Unidos, em golfinhos-roazes em regime de cativeiro ou semicativeiro, com variadas apresentações clínicas.

Um total de cinquenta e cinco (n=55) amostras foram analisadas recorrendo a rt-PCR, incluindo amostras de 5 espécies de cetáceos (*Delphinus delphis, Tursiops truncates, Phocoena phocoena, Stenella coeruleoalba* e *Kogia breviceps*). As amostras correspondiam a arrojamentos ocorridos entre os anos de 2012 a 2021, não tendo sido detetadas quaisquer amostras positivas. O movimento, dispersão e prevalência deste vírus na natureza permanece desconhecido.

Keywords: One Health, Cetáceos, Poxvirus, Coronavírus, Portugal, Rastreio.

Abstract

Virologic survey in stranded cetaceans from northern Portugal – insights on Cetacean poxvirus and Cetacean coronavirus

With an extended coastline, Portuguese waters harbor different resident and migratory species. It also registers a substantial amount of strandings every year (approximately 236 per year), which, under the supervision of ICNF, enabled the creation of a Marine Animal Tissue Bank, where tissue samples are stored for posterior analysis.

Cetacean poxvirus is responsible for poxvirus skin disease, (pinhole or ring-like lesions) and tattoo skin disease. Phylogenetically 6 species are now identified (CePV1-6). Although this virus is not connected with high mortality rates, it may be lethal to neonates and calves without maternal immunity, severely impairing small isolated populations.

To evaluate Poxvirus genetic diversity in skin lesions macroscopically compatible with Poxvirus, 47 samples collected between 2011 and 2015, from 4 different species of dolphins (*Delphinus delphis*, *Tursiops truncates*, *Phocoena phocoena* and *Stenella coeruleoalba*) were analyzed using a conventional PCR with a set of degenerate primers, previously designed for cetacean poxvirus screening. The positive samples (n=28, 58.33%) were further tested for the DNA polymerase gene, yielding 19 (40.4%) positive samples. After direct sequencing, a phylogenetic tree was designed based on the amino-acidic sequences allowing the discrimination of 3 genetic clusters, genetically closer with CePV-1, CePV-5, CePV-3 and CePV-6. An unassigned cluster was also identified, suggesting the presence of a seventh subgroup (CePV-7).

Contrary to cetacean poxvirus, *Cetacean coronavirus* was recently identified and included in the Gammacoronavirus genera. Since its discovery in 2008, cetacean coronavirus was detected in Hong Kong and United States in captives or semi-captive bottlenose dolphins presenting anorexia, diarrhea and lethargy. The movement, dispersion, and incidence of this virus in the wild is still unknown. It is important to highlight the taxonomic assignment of this virus in the Gammacoronavirus genera, which previously included coronavirus affecting strictly birds.

A total of fifty-five (n=55) samples were analyzed, collected from 5 cetacean species (*Delphinus delphis*, *Tursiops truncates*, *Phocoena phocoena, Stenella coeruleoalba and Kogia breviceps*), between 2012-2021. The viral screening was performed with a pancoronavirus rt-PCR assay, due to the unavailability of a specific tool. However, no positive samples were detected. The distribution, evolution and prevalence of this vírus remains unknown.

Keywords: One Health, Cetacean, Poxvirus, Coronavirus, Portugal, Survey.

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List of abbreviations

FMV – Faculty of Veterinary Medicine
MIMV – Integrated Master degree in Veterinary Medicine
CRAM – Centro de Reabilitação de Animais Marinhos
INIAV – Instituto Nacional de Investigação Agrária e Veterinária
ICNF – Instituto da Conservação da Natureza e das Florestas
BTAM – Banco de Tecidos de Animais Marinhos
SPVS – Sociedade Portuguesa de Vida Selvagem
IUCN – International Union for Conservation of Nature
EID – Emerging Infectious Diseases
VU – Vulnerable
EN – Endagered
CR – Critically Endagered
CDC – Centres for Disease Control and Prevention
CePV – Cetacean Poxvirus
SARS-CoV – Severe Acute Respiratory Syndrome Coronavirus
MERS-CoV – Middle East Respiratory Syndrome Coronavirus
BdCoV – Bottlenose dolphin Coronavirus
CoVs – Coronaviruses
CetPox – Cetacean Poxvirus
CetCoV – Cetacean coronavirus
CoV – Coronavirus
ORF – Open reading frame
S – Spike
N – Nucleocapsid
M – Membrane
E – Envelope
DNA – Deoxyribonucleic Acid
RNA – Ribonucleic Acid
cDNA – Complementary DNA
DNA pol -DNA polymerase
PCR – Polymerase Chain Reaction
ºC – Celsius degree
ng – Nanogram
nm - Nanometre
µL – Microlitre

mL – Millilitre

mm – millimetres

min – minutes

s – seconds

rpm – Rotations per Minute

bp – Base Pairs

UV light – Ultraviolet light

DD – *Delphinus delphis*

TT – *Tursiops truncates*

PP – *Phocoena phocoena*

SC – *Stenella coeruleoalba*

BLAST – Basic Local Alignment Search Tool

Internship activities

A curricular internship is part of the study plan for a Master's degree in Veterinary Medicine at the Faculty of Veterinary – University of Lisbon. The internship that ended in the elaboration of this project, took place within the period of January 2021 to October 2021.

The internship begun at the Centre of Rehabilitation of Marine Animals (Centro de Reabilitação de Animais Marinhos – CRAM) in Aveiro, northeast Portugal, intimately related with the North Marine Animal Stranding Network, working together in the rescue and rehabilitation of stranded animals. This first clinical internship, with the total duration of three months (1st of January – 29th March), allowed the direct contact with different marine species. Daily clinical rotations included activities such as animal manipulation and restraint, execution and interpretation of basic blood work, execution of complementary diagnostic exams, pharmacotherapy and feedings.

During this period, under the supervision of Prof. Ana Duarte and Dra. Marisa Ferreira, coordinator of the regional stranding network and of SPVS, the project started to be built. The Stranding Network secured the collection of materials for the implementation of the present virologic survey. Additional data and necropsy details were also made available by the same institution.

Laboratorial work

The laboratorial work was developed in the Virology and Molecular Biology Laboratory, of the Faculty of Veterinary Medicine (FMV) – University of Lisbon, from April to August. The remaining tasks were later finished at INIAV – Nacional Institute of Agricultural and Veterinary Research (Instituto Nacional de Investigação Agrária e Veterinária, I.P.), during the months of September and October, 2021.

Several techniques and skills were acquired during this experimental period such as: DNA extraction, DNA quantification, PCR assays (Real-time PCR, Conventional PCR), plasmid recombinants construction, agarose gel electrophoresis, DNA purification, Sanger sequencing and phylogenetic analysis.

1. Literature Review

1.1. Conservation Medicine

Conservation medicine appears as a concept in 1995, linking subjects such as Health and Ecology together. This relatively new area studies the interactions between pathogens, diseases, species and ecosystems. With its wide spectrum of subjects, the interdisciplinary nature of conservation medicine requires the cooperative work of professionals from different areas such as veterinary, biology, human medicine, public health, epidemiology, amongst others. Ecological health is seen as one of the primary goals, once “the health of all beings is connected to the ecosystem (or environments) in which they live” (Aguirre et al. 2002) (Figure 1).

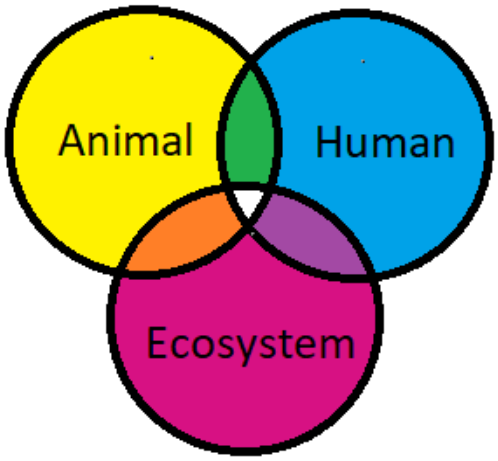


Figure 1. Conservation medicine pillars. Original

Conservation medicine strives to understand health in an ecological context (identifying what affects health on our surroundings) and uses that knowledge to create preventive or corrective approaches and to maintain the health of all species in a sustainable fashion. (Aguirre et al. 2009)

1.2. One Health

According to the definition given by the CDC, National Center for Emerging and Zoonotic Infectious Diseases, One Health is the “Collaborative, multisectoral, and transdisciplinary approach, with the goal of achieving optimal health outcomes, recognizing the interconnection between people, animals, plants, and their shared environment” (Centers for Disease Control and Prevention 2022). This concept trusts the joint work of multiple health science professions, together with their disciplines and institutions, to work on a local, national, and global scale, to promote Health. (Atlas 2012).

From a biological point of view, domesticated and wild animals should be envisaged as close human relatives. Like our species, animals share the capacity of transmitting infectious organisms between them and to us. This is the reason why we should consider our relationship with animals an open bridge for pathogens to cross, creating opportunities for their emergence in new geographic areas and communities (Zinsstag et al. 2010).

1.3. Emerging Infectious Diseases

Emerging Infectious Diseases (EIDs) are considered one of the biggest threats to general public health and several guidelines are available to help identify EID amongst other affections. We can consider an EID when a disease has recently expanded its geographic range, jumped an interspecies barrier, increased severity, suffered changes on its pathogenesis, or is caused by evolved pathogens (Kilpatrick and Plowright 2004).

Over the last decades, it was possible to detect a substantial growth on the emergence of new infectious diseases. Their flux nature combined with the continuous anthropogenic changes imposed on the environment; combine the optimal conditions for the emergence of novel zoonotic diseases. Some of them represent low rates of incidence but high fatality; others turn into pandemics with higher incidence and lower fatality rates (Daszak et al. 2004). The increased frequency of spillover events also increases the probability of the emergence of better adapted and highly transmissible pathogens (Wang and Crameri 2014).

Due to the obvious relation between ecosystem changes (toxic pollutants, introduction of species, urbanization and climate change) and disease emergence and transmission, there is a growing interest on health and environment interaction. The complexity of these interconnections encourages novel strategies for disease prevention, environmental management and conservation (Aguirre et al. 2009b).

1.4. Sentinel species

According to the Committee on Animal as Monitors of Environmental Hazards, 1991, a sentinel species should respond to several conditions. Preferably, a sentinel species should have: a measurable response to the agent or class of agents in study (e.g. accumulation of residues on tissues); a territory or home range that overlaps the area to be monitored; be easily counted and captured; and have a sufficient population size and density to permit enumeration (National Research Council, 1991).

Because this concept depends on population monitoring, it is highly useful to have updated reference populations, making animal census a highly convenient routine (National Research Council, 1991).

Other traits have also been considered throughout the years and currently the eligibility of a sentinel species requires additional characteristics (Table 1) (Basu et al. 2007).

Table 1. Desirable characteristics of a sentinel specie. Adapted from Basu et al. 2007

Characteristics of sentinel species
Widespread distribution
High trophic status
Ability to bioaccumulate pollutants
Maintained and studied in captivity
Captured in sufficient numbers
Restricted home range
Well-known biology
Sensitive to pollutants

Summarizing, sentinel species are the ones that are proved scientifically useful for the study of general health (Basu et al. 2007).

1.4.1. Cetaceans as sentinel species

The use of marine animals as sentinel organisms provides a mean to analyze diverse aquatic ecosystem (Bossart 2011). The unique adaptations of marine mammals to their environments makes them outstanding reflectors of ecosystem variations or degradation (Moore 2008). An efficient study of these animals, allows the precocious detection of potential negative human impacts on animal health, which on the other way, allows the management of such impacts (Bossart 2011).

At the end of the last century, awareness on ocean health and the conservation status of some coastal species (e.g., *Tursiops truncates*) encouraged the study of the interconnections between top predators and their respective environment. Marine mammals, like cetaceans, allow scientists to look at ocean ecosystems from top to bottom (e.g., studying bioaccumulation) and from bottom up (eg., studying distribution patterns) (Moore 2008).

Cetaceans are thus considered a sentinel group. This makes their monitoring, the assessment of their population health and the study of eventual emergent infectious diseases, extremely valuable (Bossart 2011). Having a relatively long-life span, living in coastal areas (e.g., *Phocoena phocoena)*, being carriers of a unique fat storage (blubber) able to accumulate diverse substances, and occupying a high position on the food-chain, makes these marine mammals prime sentinels (Bossart 2011). Also due to their emblematic status, cetaceans are more likely to be the object of human observation

(Moore 2008), promoting good monitoring chances. Sharing coastal areas with humans and consuming food from the same source, may also serve as an extra advantage when considering this species as strong sentinels for public health issues (Bossart 2010).

Increasing environmental pressure on marine mammals such as cetaceans, may be in the origin of more frequent epizootics, potential zoonotic pathogens' dissemination, and increases of infectious illnesses worldwide. Their utility as sentinels for the detection of (re)emerging infectious and neoplastic diseases, as for anthropogenic toxins or nefarious algal blooms, is undeniable. Whether it is a question of public health or a matter of environmental distress syndrome, cetaceans are proving to be a highly informative tool (Bossart 2011).

1.5. Cetacea

1.5.1. Taxonomy

The taxonomic order cetacea is a branch of obligate aquatic mammals (Thewissen and Williams 2003), which includes several species of mammals, previously evolved from a carnivorous stock of ungulates (50 million years ago) (Würsig 1989) (Figure 2). On current days, the largest known cetacean is the Blue Whale (*Balaenoptera musculus*), averaging 24m in length and 115.000 Kg in weight; And the smallest is the Vaquita (*Phocoena sinus*), with a medium length rounding 1.4m and weighing under 40 Kg (Thewissen and Williams 2003).

The substitution of the external hind limbs for a flat tail (fluke – main propulsive organ), modification of their forelimbs to form a paddle (flipper – mainly for steering), a streamlined body shape, and the development of blubber, marks their complete adaptation (Thewissen and Williams 2003). Their blubber, a specialized fat tissue, allows cetaceans to have diverse ranges, even thriving in Polar Regions, and to fast during their migration period. The order Cetacea is divided in two suborders: odontocetes (toothed whales) and mysticetes (baleen whales). Baleen plates, originated from a keratinous matter, were developed on the course of the evolutionary process and, combined with disproportionately large mouths, form the perfect mechanism for prey retention (Würsig 1989).

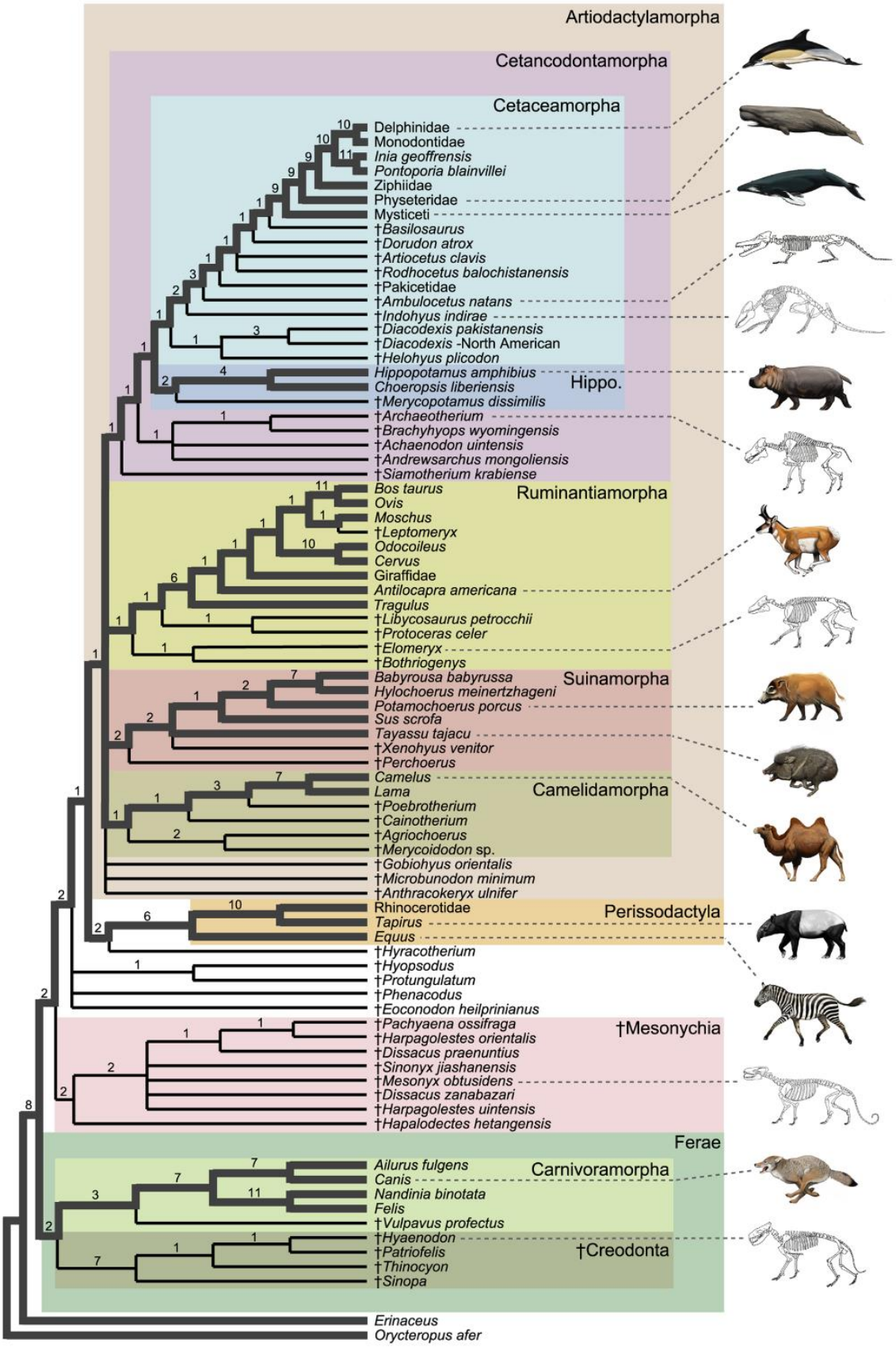


Figure 2. Cetacean evolution. Phylogenetic tree containing 12 taxonomic groups, including the Cetaceamorpha. Source: Spaulding et al. 2009

Large whales are usually migratory animals, changing locations according to the time of the year (season) and food availability. During winter, they tend to dislocate on low-latitudes mating and calving, whereas in summer, they tend to move to higher latitudes, searching for feeding grounds (Würsig 1989).

In general, cetaceans are gregarious mammals. The factors that influence the groups' size and structure are yet to be understood. However, some of the reasons behind their grouping behavior include foraging strategies, protection from predators, sexual and social interactions and raising of younglings (Marx et al. 2016).

As mentioned above, cetaceans have a vast latitudinal and longitudinal range, being present in all oceans (tropical to polar), but also an elevational range, with deep diving species like the sperm whale (*Physeter macrocephalus*), holding the utmost elevational range in the mammal kingdom, capable of reaching 1 km below sea surface (Thewissen and Williams 2003).

In conclusion, modern cetaceans represent an essential part of the ocean ecosystems, as top predators, as largescale nutrient distributers and as food source for deep-see organisms (Marx et al. 2016).

1.5.2. Cetaceans conservation

Throughout the years, cetaceans' populations (whales, dolphins and porpoises) have been severely impaired due to interaction with human activities. Besides direct hunting (whaling) for oils and meat obtainment, cetaceans are often a bycatch of high-sea as well as coastal fisheries (IUCN/SSC Cetacean Specialist Group 1994). The present climate changes are also an important factor to consider (MacLeod 2009). The rising of water's temperature directly endangers species with restricted demographic distribution (susch as *Phocoena sinus* or several river dolphins), physically unable to migrate to find cooler waters. Climate changes can also indirectly affect many species, lowering food availability and quantity, creating competition between species previously living in different areas, modifying migratory routes and ranges, impairing reproductive success, increasing interactions with human, amongst others (Simmonds and Eliott 2009).

The continuous exposure of cetaceans to sub-lethal stressors (habitat contamination, noise) caused by human activities, has a notorious impact on cetaceans' conservation. Therefore, new strategies and study approaches are being developed in order to evaluate the long-term influence of theses stressors on the species survival rate. Aspects such as physiological and behavioral changes, capable of directly interfere in species reproducibility, can be successfully used on the assessment of individual and population's health, under an ecological context (Derous et al. 2020).

A relevant obstacle faced by the scientific community, is the difficulty on the monitoring of marine megafauna. Monitoring wildlife *in loco* is *per se* an expensive activity, in which several problems must be considered such as: the high cost of sea expeditions, the large oceanic areas to cover, the animals' high mobility, the need for specialized manpower, to list some of the main identified setbacks (Peltier et al. 2014) (Table 2).

Table 2. Main monitoring techniques used on cetaceans: advantages and disadvantages. Source: Evans and Hammond 2004.

METHOD	ADVANTAGES	DISADVANTAGES
Survey platforms/ headland	• Inexpensive (generally) • Not labor intensive • Non-intrusive	• Information that requires close proximity to animals is hard to collect • Coverage of small detection areas (installation sight)
Vessel	• Coverage of wider areas over longer periods of time (depending on vessel) • Biological samples be collected • Environmental information can be collected	• Large vessels can be expensive and may require a bigger laboring unit • Small vessels are limited in terms of area coverage
Aircraft	• Large areas covered in a shorter period of time • Efficient use of windows of good weather • Not labor intensive	• Collection of samples (biological and environmental) is limited • Logistical limitations (space) • Expensive to charter regardless the time required

A valid alternative to the direct observation of populations, is the study of cetaceans' stranding. When monitored correctly, cetacean strandings are an important source of information and insights on populations' status, availability of biological samples and threats' assessment, allowing the study of abnormalities affecting marine ecosystems (Ijsseldijk, Brownlow, and Mazzariol 2019).

Acording to the IUCN Red Book, 24,4% of cetacenas species are now under the conservation status of Vulnerable (VU), Endangered (EN) or Critically Endangered (CR) (Figure 3). Also, nineteen species are presently seeing their populations' numbers decreased (*Lipotes vexillifer, Cephalorhynchus hectori, Phocoena sinus, Sotalia fluviatilis, Orcaella brevirostris, Eubalaena glacialis, Neophocaena phocaenoides, Neophocaena asiaeorientalis, Orcaella heinsohni, Cephalorhynchus eutropia, Pontoporia blainvillei, Sousa sahulensis, Mesoplodon perrini, Berardius minimus, Inia*

geoffrensis, Mesoplodon stejnegeri, Sousa plumbea, Sousa chinensis, Sousa teuszii). (The IUCN Red List of Threatened Species 2021 Jan 5).

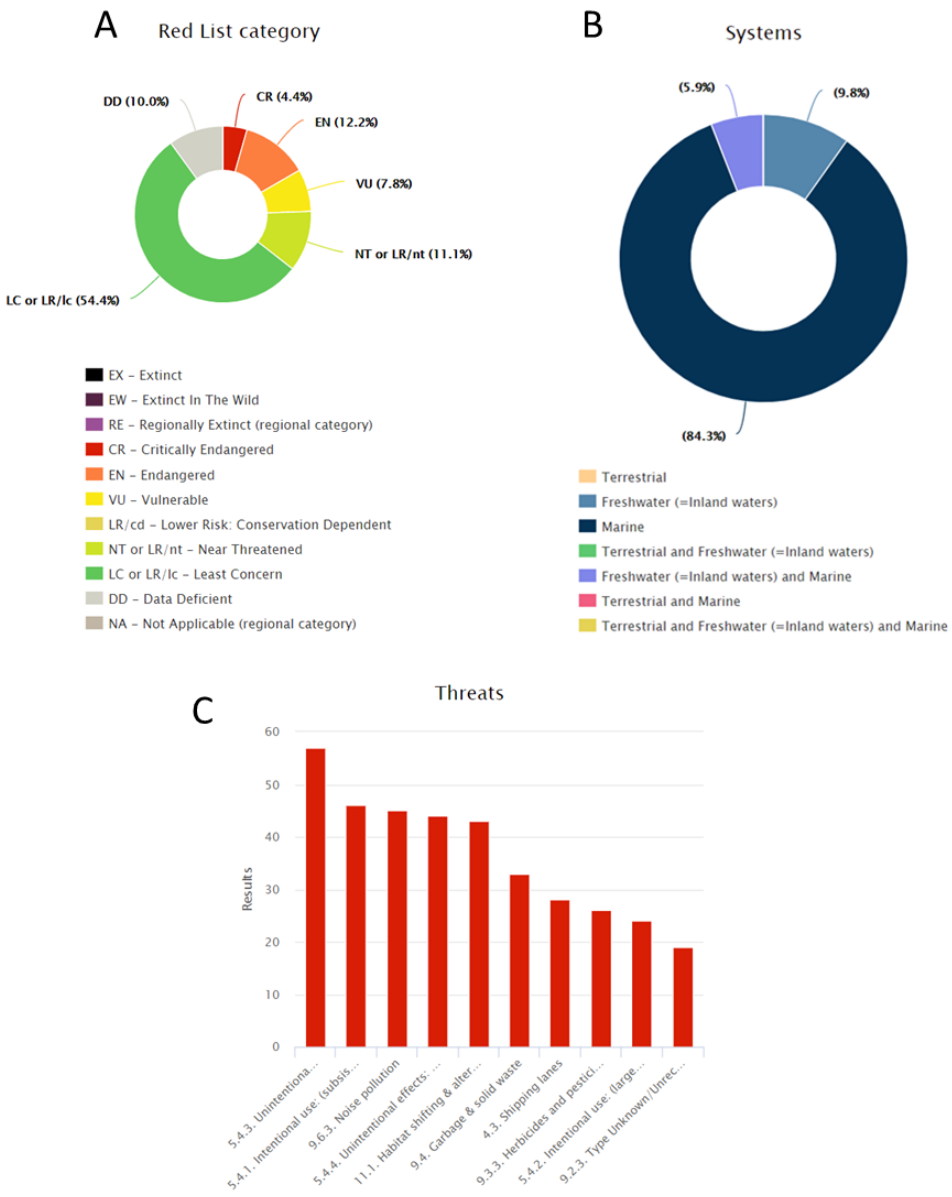


Figure 3. IUCN Red list data – Cetacean conservation status. (A) Distribuition of species according to conservation status; (B) Habitat occupied by cetacean species; (C) Main threats against cetaceans' conservation. Source: https://www.iucnredlist.org/search/stats

1.5.3. Cetacean Conservation status in Portugal

Between the years of 2011 and 2017, the project "Conservation of Cetaceans and Seabirds in Continental Portugal, LIFE+ MarPro project", with innumerous collaborators from national and foreign partner organizations, was implemented. The project resulted in the publication of two relevant census: cetaceans and seabirds. Data

was obtained through direct observation (coastal, areal and boat) but also from stranding occurrence along the Portuguese coastline. To the present dissertation, only the cetacean census was taken into consideration (Table 3).

Table 3. Cetacean species observed in continental Portugal and correspondent conservation status. Source: Vingada and Eira 2018

Common name	Scientific name	Occurrence	Conservation status
Mink whale	*Balaenoptera acutorostrata*	Resident	Vulnerable
Sei whale	*Balaenoptera borealis*	Occasional	Not rated
Bryde's whale	*Balaenoptera edeni*	?	Not rated
Blue whale	*Balaenoptera musculus*	?	Not rated
Fin whale	*Balaenoptera physalus*	Migrator	Not rated
North Atlantic right whale	*Eubalaena glacialis*	?	Not rated
Humpback whale	*Megaptera novaeangliae*	Occasional	Not rated
Common dolphin	*Delphinus delphis*	Resident	Least concern
Short-finned pilot whale	*Globicephala macrorhynchus*	Occasional	Insufficient information
Long-finned pilot whale	*Globicephala melas*	Resident	Insufficient information
Risso's dolphin	*Grampus griseus*	Resident	Insufficient information
Pygmy sperm whale	*Kogia breviceps*	Resident	Insufficient information
Dwarf sperm whale	*Kogia sima*	?	Not rated
Fraser's dolphin	*Lagenodelphis hosei*	?	Not rated
Atlantic white-sided dolphin	*Lagenorhynchus acutus*	?	Not rated
White-beaked dolphin	*Lagenorhynchus albirostris*	?	Not rated
Sowerby's beaked whale	*Mesoplodon bidens*	?	Not rated
Blainville's beaked whale	*Mesoplodon densirostris*	?	Not rated
Gervais's beaked whale	*Mesoplodon europaeus*	?	Not rated
True's beaked whale	*Mesoplodon mirus*	?	Not rated
Orca	*Orcinus orca*	Migrator	Insufficient information
Harbour porpoise	*Phocoena phocoena*	Resident	Vulnerable
Sperm whale	*Physeter macrocephalus*	Occasional	Not rated
False killer whale	*Pseudorca crassidens*	Occasional	Not rated
Striped dolphin	*Stenella coeruleoalba*	Resident	Least concern
Atlantic Spotted Dolphin	*Stenella frontalis*	?	Not rated
Common bottlenose dolphin	*Tursiops Truncatus*	Resident	Least concern
Cuvier's beaked whale	*Ziphius cavirostris*	Resident	Insufficient information

With an extended coastline, Portuguese waters harbor different resident and migrant species, crossing the Atlantic shores during migration. Cetaceans' species such as the stripped dolphin (*Stenella coeruleoalba*), bottlenose dolphin (*Tursiops truncates*) and fin whales (*Balaenoptera physalus*) are frequently seen across the Portuguese

coastal areas, but also *offshore* (Vingada and Eira 2018). At the end of the census, 28 different species were recorded (21 Odontocetes and 7 Mysticetes), including 5 species never before observed in Portuguese continental waters (Vingada and Eira 2018).
During the survey, the main threats to the conservation of these animals were also studied. Renewable energy production, fisheries, pollution (plastics, oil spills, heavy metals, ghost nets), lower food availability, introduction of diseases and parasites were some of the main identified threats.

1.5.4. Portuguese National Marine Animal Strandings Network

Every year, numerous episodes of stranding occur, due to anthropogenic (pollution, fisheries and related activities, maritime traffic) and non-anthropogenic (natural, pathogens related, biotoxins) causes (Díaz-Delgado et al. 2018).
Marine mammal stranding networks exist throughout the world, including in China (Liu et al. 2019), France (Peltier et al. 2019), and in the United States of America (Gulland and Hall 2007), amongst others. Stranding networks, governmental authorities or non-governmental organizations have developed unique standard stranding responses, creating necropsy protocols to document and track stranded marine mammal demographics, signaling and health data (Chan et al. 2017).

In continental Portugal, a substantial amount of stranding occurs (approximately, 236 per year) (Vingada and Eira 2018), which led to the creation of a National marine animal stranding network in 1979 (Marçalo et al. 2021). Presently, under the coordination of ICNF (Instituto da Conservação da Natureza e Florestas), the network is subdivided in four regional fronts: North, South, Center and, most recently, Alentejo. Cadavers are collected and necropsied in proper conditions, by the regional stranding networks. Main macroscopic alterations are recorded and tissue samples are collected for posterior study and analysis. Throughout the years, this resulted in the assembly of a vast tissue bank. Diverse materials are stored for different purposes including histopathology, genetics, virology/bacteriology, diet assessment, contamination assessment, amongst others (Vingada and Eira 2018).

The fact that only a small fraction of dead cetaceans strand makes the detailed study of these last an essential feature to comprehend the populations' stance (Peltier et al. 2012).

The field of marine mammal medicine has developed considerably over the last 10 years (Gulland and Hall 2007). Efforts to minimize death and suffering have also improved, since an increasing number of stranding network facilities employ veterinarians to advise on medical care of stranded marine mammals.

1.6. Cetacean virology

In the last years, several multifactorial diseases with complex etiologies have been described in marine mammals (Bossart 2010). A considerable proportion of these diseases are associated with novel viral infections (Van Bressem et al. 2009). It is known that viruses represent the most abundant members on marine ecosystems, being estimated that the oceans together, may contain an amount of virus particles in the order of 10^{30} (Munn 2006).

New viruses are now frequently identified, such as a novel gammaherpesvirus, found in sexually mature stranded California sea lions, with urogenital cancer, (otarine herpesvirus-1); Also, transmissible orogenital papillomatosis on bottlenose dolphins (*Tursiops truncates*), occasionally related with metastatic squamous cell carcinoma, was found to be caused by a newly sequenced papillomaviruses (TtPV-1, TtPV-2) in association with a novel herpesvirus (Bossart 2010).

Studies indicate that the emergence and severity of some new viral diseases is related to chemical or biological contamination of the surrounding environment (Christon J. Hurst 2011). Extrinsic anthropogenic factors (biological, acoustic and chemical pollution, climate change, fisheries, and heavy boat traffic), have the capacity to disturb virus–host equilibrium. These disruptions may be observed through and on different aspects when assessing an ecosystem: reduction in the number of animals of a population and, therefore, decreased probability of an enzootic successful establishment; decrease on the population's immune response; depressed food supplies; increased stress factors (Christon J. Hurst 2011).

Depending on the agent, different strategies are adopted to ensure the virus maintenance in the environment (Christon.J. Hurst 2011) (Table 4).

Table 4. Assorted viral strategies to perpetuate on host populations. Adapted from: C.J. Hurst 2011

Virus	Strategy
Morbilliviruses	Maintaining a population threshold, with the goal of maintaining the virus endemic;
Papillomaviruses	Sexual transmission;
Herpesviridae	Capable of activate a state of latency in their host, reactivating under specific stimuli;
Endogenous retroviruses	Integration on host genome;
Influenza A	Nonspecific reservoir (miscellaneous unrelated species);
Caliciviruses	Nonspecific reservoir (miscellaneous unrelated species).

To date, viruses belonging to at least 10 different families were identified in marine mammals (Table 5). However, clinical features, pathology and epidemiology of the diseases are not always clear.

Table 5. Summary of the virus identified in marine mammals until the current days. Adapted from Van Bressem et al, 1999

Family	Name	Pathology	Host range	Distribution	Source
Paramyxoviridae	Cetacean morbillivirus	Pneumonia, Encephalities, Lymphoid depletion	Cetaceans	North Atlantic, Mediterranean, South Pacific, Black Sea	Kennedy et al. (1998), Domingo et al. (1990), Van Bressen et al. (1993a), Visser et al. (1993), Lipscomb et al (1994a,b), Di Guardo et al. (1995), Duignan et al. (1995a,b), Blinxerkrone-Moller et al. (1996), Tsur et al. (1997), Birkun et al. (1998), Reidarson et al. (1998), Van Bressen et al. (1998a,b)
Poxviridae	Cetacean poxviruses	Cutaneous lesions (ring, tattoo)	Cetaceans	North Atlantic, Southeast Pacific	Flom & Houk (1979), Geraci et al. (1979), Van Bressen et al. (1993b), Van Bressen & Van Waerebeek (1996)
Papovaviridae	*Phocoena spinipinnis* papillomaviruses, Other papillomaviruses	Genital warts, Cutaneous warts	Cetaceans	Southeast Pacific, North Sea	Van Bressen et al. (1996), Cassonnet et al. (1998), Van Bressen et al. (1999)
Herpesviridae	Herpes like viruses, Porpoise herpesvirus	Skin disease, Encephalitis	Cetaceans	St. Lawrence Estuary, Churchill River, Southeast Pacific, North Sea	Martineau et al. (1988), Barr et al. (1989), Kennedy et al (1992b), Van Bressem et al. (1994)
Orthomyxoviridae	A/Whale/Maine/1/84 A/Whale/Maine/2/84 A/Whale/PO/19/76	Unknown	Cetaceans Seabirds Others(?)	Northwest Atlantic, South Pacific	Hinshaw et al. (1986), Lvov et al. (1978)
Rhabdoviridae	Dolphin rhabdovirus-like virus	Unknown	Cetaceans, Pinnipeds, Others(?)	Northwest Europe, Mediterranean	Osterhaus et al. (1993)
Calicivirdae	San Miguel sea lion virus, Vesicular exanthema of Swine virus, Cetacean calicivirus, Walrus calicivirus, Mink calicivirus	Vesicular skin lesions, Unknown	Mammals, Fishes, Trematodes, Nematodes, Others(?)	North Pacific, Arctic	Smith & Latham (1978), Smith et al. (1983), Smoth et al. (1987), Smith et al. (1990)
Hepadnaviridae		Chronic persistant hepatitis	Delphinidae	Unknown	Bossart et al. (1990)
Adenoviridae		Unknown	Cetaceans	Arctic, Antarctic	Smith & Skilling (1979), Smith et al. (1987), De Guise et al. (1995)
Coronaviridae	Beluga whale coronavirus SW1, Bottlenose dolphin coronavirus	Chronic hepatitis, Pulmonary disease	Cetaceans	Hong Kong (captive), USA (captive)	Mihindukulasuriya et al. (2008), Woo et al. (2014)

For many years, the roll of virus in marine ecosystems was underrated, until the morbillivirus up rise, which lead to a severe impairment of the populations growing rates. This event was responsible for mass mortalities of harbour porpoises (*Phocoena phocoena*) in the northeastern Atlantic (1988-1990) and striped dolphins (Stenella coeruleoalba) in the Mediterranean in (1990-1992) (van Bressem et al. 1999). Currently, the virus remains highly active, being frequently identified on periodic mortality events, epidemics or endemically. The virus was able to reach a wide geographical range, being present in the Atlantic, Pacific and Indian oceans, and in several seas (Van Bressem et al. 2014). Also in Portugal, the detection of Dolphin Morbillivirus was confirmed (Bento et al. 2016), but unrelated with disease or mortality events in the Portuguese coastline.

1.6.1. Poxvirus

Poxviridae is a family of complex DNA viruses that replicate on cells' cytoplasm (Fields and Howley 2001). All *Poxviridae* members replicate in skin and mucosa cells, inducing localized or generalized lesions, according to the host and the virus (Blacklaws et al. 2013).

With a linear double-stranded DNA, their genome length can vary from 130 kbp (parapoxviruses) to around 230 kbp (avipoxviruses). These viruses have unique biologic properties and have demonstrated their capacity to severely affect human health. Two poxviruses: variola virus and molluscum contagiosum virus, are obligate human pathogens; others can jump the interspecies barrier, animal to human. The *Poxviridae* family includes two subfamilies: *Chordopoxvirinae*, which has vertebrate hosts; and *Entomopoxvirinae*, affecting invertebrates. The *Chordopoxvirinae* is divided on eight genera: *Orthopoxvirus, Avipoxvirus, Parapoxvirus, Suipoxvirus, Capripoxvirus, Molluscipoxvirus, Yatapoxvirus and Leporipoxvirus* (Fields and Howley 2001).

The poxviruses are unique in the way they entry cells. Current opinion accepts the production of two types of infectious particles: mature virions (MV) and extracellular virions (EV), which have an additional outer membrane disrupted prior to fusion (Moss 2012). These two particles do not share common viral surface epitopes (Schmidt et al. 2012). The initial infectious form – MV – has a single external membrane and, although some of these forms can remain free or in inclusions within the cytoplasm until liberated, others enter a different pathway. Here, a modified trans-Golgi or an endosomal membrane involves the pre-existent particle, forming a triple-membrane. This set is called wrapped virion (WV). The newly formed particle approaches the periphery of the host cell (mediated by microtubules) and finally, a fusion occurs between the outer membrane and the plasma membrane, releasing an extracellular enveloped virion – EV (Moss 2012).

The high number of proteins used by poxviruses when entering cells is unprecedented, which may explain their ability to infect a wide range of cells (Moss 2012). Poxviruses infect the host mainly through the skin's cornified epithelium or the mucosal surface. The infection is more likely to occur through localised abrasions, allowing the virus to access the epidermal and dermal layers. In these layers, the virus finds the target cells to replicate and to rapidly spread to the draining lymph node, via lymphatics or infected cells (Born et al. 2000).

In general, poxviruses show species specificities that range from narrow to broad, but the mechanisms behind the regulation of host tropism of individual poxviruses is still not clear. From a vast collection of open reading frames (ORF) present on poxviruses' genome, at least 90 are specifically conserved among them, and are responsible for essential functions such as replication and morphogenesis. The remaining ORFs, due to adaptive evolution, end up diverging between different poxviruses, and are called non-conserved genes. These genes control the host range of each virus, immunomodulation and pathogenesis (McFadden 2005). Poxviruses do not require specific receptors to enter cells, on the contrary the host range and virus specificity rely on the capacity that the virus have to regulate subsequent events in the infected cell (recruiting transacting factors, inhibiting cellular antiviral responses – apoptosis, interferon pathway) (McFadden 2005). Latest studies have started to identify some of the proteins involved in these complex processes: E3 proteins, K3 family proteins and eIF2α homolog (Cao et al. 2020).

Several poxviruses' genomes have been sequenced, including the human Poxvirus such as smallpox, vaccinia, and monkeypox with severe consequences in human health, and some exotic poxviruses such as camelpox or lumpy skin disease viruses (Bracht et al. 2006). Most recently, the Cetacean Poxvirus' complete genome was also successfully sequenced and published (Rodrigues et al. 2020).

1.6.2. Cetacean poxvirus

Poxvirus was identified on cetaceans in 1979. Even though it had already been reported in other marine mammals (pinnipeds), its visualization under electronic microscopy, in the skin of a *Tursiops truncates*, was the first ever register on cetaceans, allowing the clear visualization of viral aggregations in the cytoplasm of infected skin cells (Geraci et al. 1979)

The cetacean poxviruses integrate a new genus of Chordopoxvirinae and are divided into two subgroups: cetacean poxvirus CePV-1, affecting odontocetes (toothed whales); cetacean poxvirus CePV-2, in mysticetes (Christon J. Hurst 2011). Due to the marked heterogenicity of the virus, Barnett et al. 2015 proposed a new organization

based on genomic differences. This led to the reorganization into 6 new subgroups: CePV-1, CePV-2, CePV-3, CePV-4, CePV-5 and CePV-6 (Figure 4)

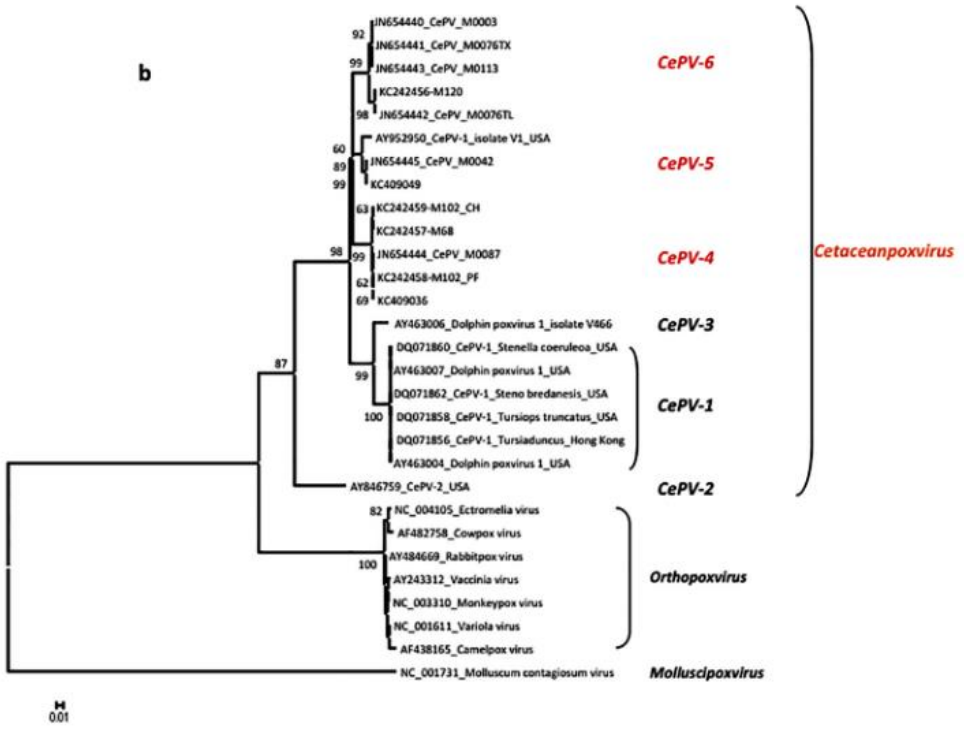


Figure 4. Cetacean poxvirus phylogenetic tree. Identification of the 6 subgroups proposed by Barnett et al. Source: Barnett et al. 2015

Poxvirus infection has been reported in a vast range of cetacean families, such as Delphinidae, Phocoenidae, Ziphiidae, Balaenopteridae and Balaenidae. Geographically, the reports reach from Europe, to America, Middle East, Australia, and New Zealand (Christon J. Hurst 2011). Cetacean poxviruses have also been identified throughout the years, mainly due to its visible macroscopic manifestations.

Two slightly different clinical presentations are described: pinhole or ring-like lesions (Fiorito et al. 2015); and tattoo skin disease (TSD) characterized by irregular, grey, black or yellowish, stippled skin lesions (Van Bressem et al. 2009) (Figure 5). Individual tattoo lesions may persist for months or years and recurrences are an existing possibility (Christon J. Hurst 2011).

Although the cetacean poxvirus is not associated with high mortality rates, it may be lethal to neonates and calves without maternal immunity, severely impairing small isolated populations (Van Bressem et al. 2009). In healthy groups, juveniles demonstrate higher prevalence of skin lesions (Blacklaws et al. 2013). This can be explained considering the loss of maternal immunity and the lack of active immunity, present in many adults following infection (Christon J. Hurst 2011). In populations with a poor health

state, adults show a higher TSD prevalence comparing with juveniles (Blacklaws et al. 2013), most probably due to immunosuppression (Christon J. Hurst 2011).

Figure 5. Macroscopic aspects of lesions caused by the Cetacean poxvirus, on a Portuguese *Tursiops truncates* population. Black tips point to dorsal tattoos. Source: Van Bressem et al. 2003

Anthropogenic factors that interfere with cetacean's ecosystem may alter the virus behavior (prevalence, clinical manifestations, recurrence capacity). For instance, environmental changes may affect the "normal" course of the disease leading to septicemia and death, or modify the classical epidemiologic pattern seen on populations where the virus is endemic (Christon J. Hurst 2011).

The perspective upon Cetacean poxviruses has suffered gradual changes with the continuous discovery of new species and clusters. Following previous studies, understanding the heterogeneity of the virus is necessary to map the virus' behavior.

1.6.3. Coronavirus

Coronaviruses are included in the family *Coronaviridae* (Fields and Howley 2001) within the order *Nidovirales*. The subfamily *Coronavirinae* is divided in four distinct genera: Alphacoronaviruses; Betacoronaviruses; Gammacoronaviruses; and Deltacoronaviruses (Payne 2017) (Table 6).

Gammacoronavirus and Deltacoronavirus are coronaviruses that have evolved from birds, with some known lineages making the jump into mammals. Alphacoronavirus and Betacoronavirus originated from bats and developed the capacity to spread to other mammals such as humans (Jacob Machado et al. 2021).

They are large (120–160 nm) (Mordecai and Hewson 2020), enveloped, RNA viruses, having the largest genome amongst the RNA viruses. Affecting both humans and animals, they are in the origin of innumerous highly prevalent diseases (Fields and Howley 2001).

The coronaviral genome encodes four major structural proteins: spike (S), nucleocapsid (N), membrane (M), and the envelope (E). All of these proteins are required to produce a structurally complete viral particle; however, the presence of all four is not utterly necessary to produce an infectious virion (Schoeman and Fielding 2019).

Coronaviruses have developed diverse strategies for the initiation of an infection. This holds true for the attachment stage, subsequent membrane fusion (Winter et al. 2006), and replication (Ziebuhr 2005). Depending on the virus and on the host cell, the proteins used for the binding process can differ; for instance, Aminopeptidase N is used by some alphacoronavirus, angiotensin-converting enzyme 2 is used by some alphacoronavirus and betacoronavirus, and glycan sialic acid is used by gammacoronavirus (Winter et al. 2006).

In addition, in coronaviruses, replication happens resorting to a unique mechanism. The mRNA synthesis occurs through a complex set of moves performed by the RNA polymerase, from one region of the genomic RNA to another, sharing a common 5' end (Payne 2017). This results in a high frequency of recombination (Murphy et al.

1999). After the assembly of the different viral proteins, virions enter the Golgi complex where maturation occurs (Hogue and Machamer 2014). Finally, after budding through the intracellular membranes, the infectious particle is released through exocytosis (Murphy et al. 1999).

Coronaviruses infection is mostly related with respiratory or enteric diseases in most host species. Neurological illness or hepatitis occurrence are a less frequent manifestation of the viral infection (Jacob Machado et al. 2021).

Members from the *Coronaviridae* family, are in the origin of several epidemics' (SARS-CoV, MERS-CoV) and recently of the present pandemic due to SARS-CoV-2 (Piret and Boivin 2020).

Table 6. Coronavirus species according to virus taxonomy in 2016. Source:.Miłek and Blicharz-Domańska 2018.

Alphacoronavirus	Betacoronavirus	Deltacoronavirus	Gammacoronavirus
Bat coronavirus HKU10	Betacoronavirus 1	Bulbul coronavirus HKU11	Avian coronavirus
Human coronavirus 229E	Hedgehog coronavirus 1	Common moorhen coronavirus HKU21	Beluga whale coronavirus SW1
Human coronavirus NL63	Human coronavirus HKU1	Coronavirus HKU15	
Miniopterus bat coronavirus 1	Middle East respiratory syndrome-related coronavirus	Munia coronavirus HKU13	
Miniopterus bat coronavirus HKU8	Murine coronavirus	Night heron coronavirus HKU19	
Mink 19oronavírus 1	Pipistrellus bat coronavirus HKU5	Thrush coronavirus HKU12	
Porcine epidemic diarrhoea vírus	Rousettus bat coronavirus HKU9	White-eye coronavirus HKU16	
Rhinolophus bat coronavirus HKU2	Severe acute respiratory syndrome-related coronavirus	Wigeon coronavirus HKU20	
Scotophilus bat coronavirus 51	Tylonycteris bat coronavirus HKU4		

1.6.4. Cetacean Coronavirus

A new species of coronavirus was recently reported in 2008: the *Cetacean coronavirus*, included in the genera *Gammacoronavirus* (Mihindukulasuriya et al. 2008). The taxonomic assignment of this virus in the *Gammacoronavirus* genera, which

previously included coronavirus affecting strictly birds (Patrick C. Y. Woo et al. 2014), highlights the recombination potential of this virus family.

The first description of Cetacean coronavirus (CoV[BWCoV] SW1) occurred on a captive Beluga whale, presenting an acute generalized pulmonary disease and terminal acute liver failure, which resulted in its death. Taking in consideration the lesions above described, a liver homogenate recovered during necropsy was used for molecular viral detection followed by direct sequencing (Mihindukulasuriya et al. 2008).

During the following years, two different and relevant variations of the virus were reported in bottlenose dolphins (Figure 6). In 2014, under a surveillance program preformed on an aquarium in Hong Kong, fecal samples were taken from pinnipeds and cetaceans, and tested for different virus. A total of three Indo-Pacific bottlenose dolphins were positive for Cetacean coronavirus (CoV [BdCoV] HKU22), also by molecular detection followed by direct sequencing. The individuals did not present any symptoms and remained asymptomatic throughout the process (Patrick C. Y. Woo et al. 2014).

In 2019, four Atlantic bottlenose dolphins belonging to the US Navy Marine Mammal Program, presented mild clinical signs such as lethargy, anorexia and diarrhea. After the acute onset of the disease, fecal samples were collected, subjected to molecular analysis and found positive for Cetacean coronavirus (US BdCoV). After the acute illness, clinical signs naturally disappeared and no lethality was registered.

Figure 6. Locations where Cetacean coronavirus was identified until the present year (2022) - San Diego and Hong Kong. Source: Google Earth

To evaluate the role of genetic features, including mutations in different genes, on the severity of clinical signs, several phylogenetic trees were designed, accessing different genomic sequences (Figure 7). The spike (S) and matrix (MA) genes were found to present interesting variations (Wang et al. 2020).

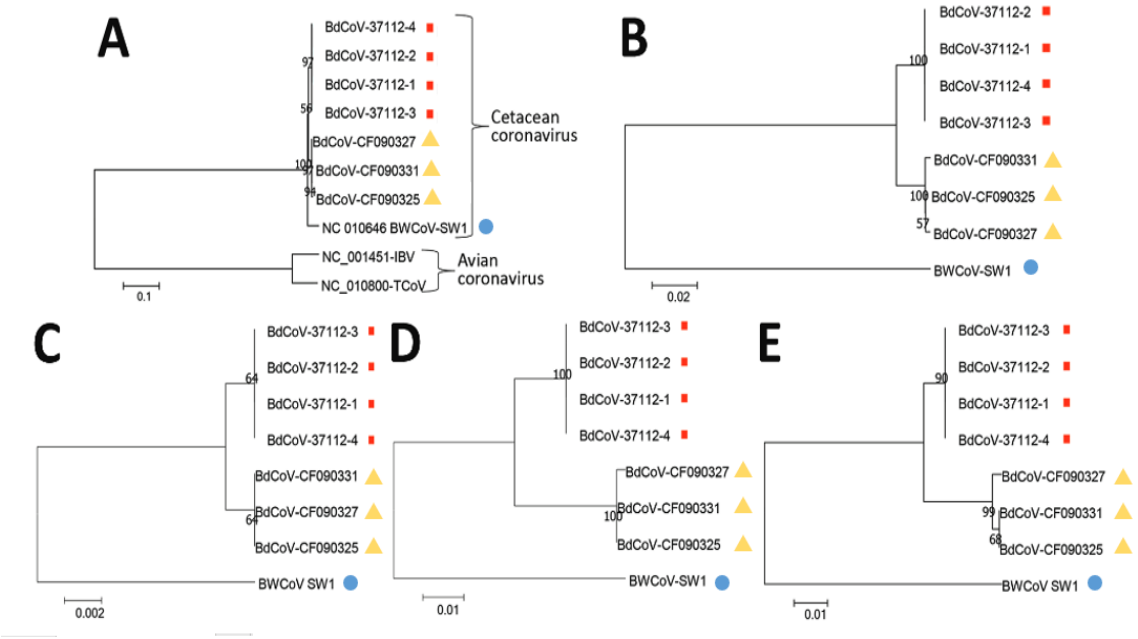


Figure 7. Phylogenetic analysis of Cetacean coronavirus by A) complete genome, B) spike, C) envelope, D) matrix and E) nonstructural protein 5a genes. The elements of this tree include BWCov (beluga whale coronavirus), represented by blue circles, and BdCoV (bottlenose dolphin coronavirus), variants from USA, with yellow triangles, and Hong Kong, with red squares. Adapted from: Wang et al. 2020

The presence, dispersion, and behavior of the cetacean coronavirus in the wild is yet unknown. Due to the zoonotic potential of coronaviruses, continuous discovery of novel CoVs, and their genomic and phylogenetic characterization is of major importance (P. C. Y. Woo et al. 2014). Additional surveillance is advisable to monitor the virus presence worldwide (Wang et al. 2020).

2. Objectives

This project aims to contribute for a wider knowledge on the genetics and pathogenic impact of both *Cetacean poxvirus* (PoxCet) and *Cetacean coronavirus* (CoVCet). Although belonging to two different major families of virus and affecting different organ systems, the parallel study of these pathogens can provide insights about the cetacean populations' health. By rolling virological tests on tissues of stranded cetaceans previously collected by the North Marine Animal Stranding Network, our aim is to collect information regarding the viruses' presence in wild environments and their prevalence in

the Portuguese continental shores, gathering important phylogeographic information. Due to the correspondent necropsy details, virus detection will be correlated with the recorded macroscopic findings.

Monitoring wildlife populations, especially sentinel species as cetaceans are known to be, is a gateway to evaluate ecosystems' stability. In addition to the assessment of the animal populations' health status, these activities enable the collection of ecological and conservation data, allowing us a unique opportunity to observe and study virus distribution in the wild. If used systematically and correctly, this kind of information can also be used in prevention of EID's and zoonotic diseases.

This project is part of the MSc thesis of the author, a MIMV student, whose supervisors were Ana Isabel Duarte (DVM, MSc, PhD), researcher at INIAV, whose main activities involve studies in virology and immunology; and Marisa Ferreira, member of Sociedade Portuguesa de Vida Selvagem (SPVS) direction board, with vast experience in marine mammal biology, ecology, and wildlife monitorization. It is important to emphasize the previous work conducted by the research team and consultants associated with this project contributing for the higher probability of success.

3. Material and Methods

3.1. Sample origin

The samples were obtained in collaboration with the Regional marine animal stranding network of northern Portugal, which covers the area between Peniche and Caminha (Figure 8). Dead stranded cetaceans are by protocol, brought to suitable facilities, where necropsies are performed.

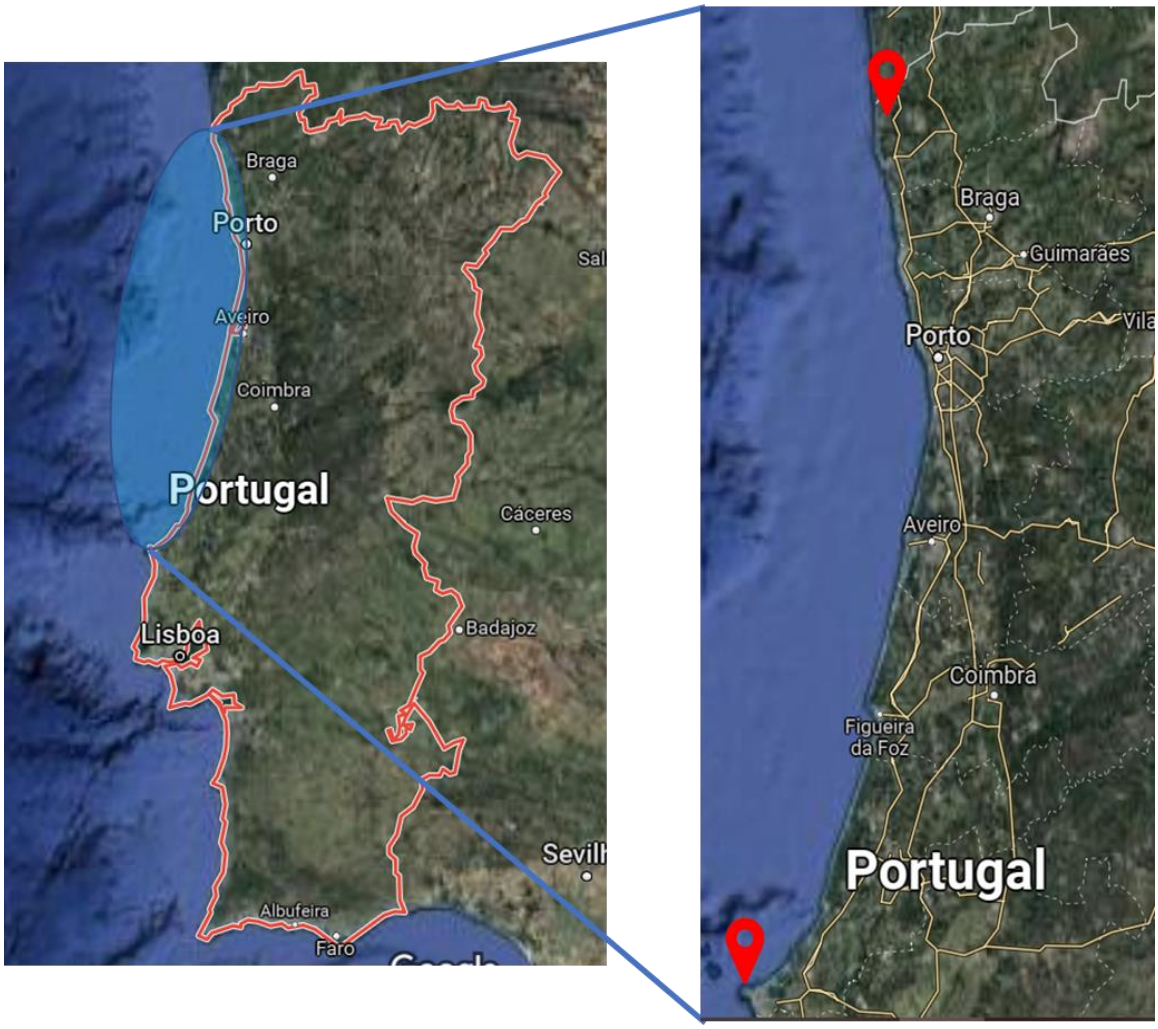


Figure 8. Work range of the Regional Marine mammal Stranding Network operating in the north of Portugal. Source: Google maps.

Data such as stranding site, body condition, state of decomposition, sex, amongst others, are registered during the procedure. Tissue samples are selectively collected and sent to immediate analyses. Samples for virological testing are preserved in RNAlater and stored at -20ºC, in the tissue bank facilities (BTAM – Banco de Tecidos de Animais Marinhos), until processed.

All necropsies followed the advised safety measures and the intervenient personnel wore suitable individual protection equipment.

3.2. Sample collection

3.2.1. Cetacean poxvirus survey

For the Cetacean poxvirus survey, a total of forty-seven (n=47) samples of skin lesions were analyzed. These included materials from four (4) cetacean species: *Delphinus delphis* (n=37), *Stenella coeruleoalba* (n=5), *Tursiops truncates* (n=1), *Phocoena phocoena* (n=4). Materials stored in the tissue bank were randomly selected, with the following temporal distribution: 2011 (n=1), 2013 (n=4), 2014 (n=41) and 2015 (n=1) and no particular body area was requested (flippers, fluke, abdominal region, jaw). Lesions were measured, characterized and photographed before collection. All samples were maintained in RNAlater (1:5), stored under a 4°C temperature for 24-48h and frozen at -20°C until manipulation.

A list of the collected materials can be found on Annexes (Table 15).

3.2.2. Cetacean coronavirus survey

Regarding the Cetacean coronavirus survey, a total of fifty-five (n=55) samples were studied, including five (5) cetacean species: *Delphinus delphis* (n=29), *Stenella coeruleoalba* (n=6), *Tursiops truncates* (n=8), *Phocoena phocoena* (n=10) and *Kogia breviceps* (n=2). Materials stored in the tissue bank were randomly selected, with the following temporal distribution: 2012 (n=8), 2013 (n=5), 2014 (n=15), 2015 (n=8), 2016 (n=5) and 2021 (n=14).

The molecular analyses were performed on pools of two organs, namely kidney and mesenteric lymph node. Collected organs, kidney and mesenteric lymph node, had no specific macroscopic alterations. Necropsy images were not requested.

All samples were maintained on RNAlater (1:5), stored under a 4°C temperature for 24-48h and frozen at -20°C until manipulation.

A list of the collected materials can be found in Annexes (Table 16).

3.3. DNA and RNA extraction

3.3.1. Cetacean poxvirus survey

Total DNA was extracted from skin samples using QIAamp® DNeasy Blood and tissue Mini Kit, according to the manufacturer's instructions for the Purification of Total DNA from Animal Tissues (Spin-Column Protocol).

Sections of 25 mg were cut from the sample, previously kept in RNAlater, and place in a 1.5 mL microcentrifuge tube. A volume of 180 µL of ATL and 20 µL of Proteinase K and a stainless-steel bead (5 mm diameter) were added to the tube. The sample was homogenized on the Tissue Lyser II™ (Qiagen) for 2 minutes at 20 Hz and, after rotation of the rack, the sample was subjected to another 2 minutes at 20 Hz, in the same equipment. After homogenization samples were incubated at 56 ºC for 3-18h. After the lysis was complete, 200 µL of Buffer AL and 200 µL of ethanol (96–100%) were added and mixed by vortexing the tube. The mixture obtained in the last step, was transferred into a DNeasy Mini spin column and centrifuged for 1 minute at 13.000xg. The flow-through was discarded. The DNeasy Mini spin column was then transferred to a new 2 mL collection tube, where 500 µL of Buffer AW1 were added. A new centrifugation was performed. Flow-through was again discarded and the DNeasy Mini spin column placed in a new collecting tube. A volume of 500 µL of Buffer AW2 was added to the column. Following the last step, a centrifugation was performed at 16.000xg, for 3 minutes, in order to completely dry the silica membrane of the column. The collection tube and respective flow-through was discarded and the DNeasy Mini spin column finally placed in a 1.5 mL microcentrifuge tube. For the elution, a volume of 100 µL of Buffer AE was directly pipetted on the membrane. One last centrifugation was done with the duration of 1 minute, at 13.000xg. The purified DNA was stored at – 20 ºC until further manipulation.
(Dneasy Blood & Tissue Handbook 2020).

3.3.2. Cetacean coronavirus survey

Total RNA was extracted from kidney and mesenteric lymph node samples using the QIAmp RNA blood minikit, according to the manufacturer's instructions.

From each sample, 20 mg were weighed and placed in a 2 Ml microcentrifuge tube. A volume of 350 µL of Buffer RLT (previously mixed with β-mercaptoethanol in the proportion of 1:100) and a stainless-steel bead (5 mm diameter) were added to the tube prepared on the previous step. The sample was homogenized on the Tissue Lyser II for 2 minutes at 20 Hz and, after rotation of the rack, the sample was subjected to another 2 minutes at 20 Hz, in the same equipment. Afterword, the tube was centrifuged for 1 minute at maximum speed and the resulting supernatant was transferred to a 1.5 Ml micro centrifuge tube. A volume of 350 µL of 70% ethanol was mixed into the lysate. The mixture (including any eventual precipitate) was carefully pipetted to a QIAamp spin column in a 2 Ml collection tube and centrifuged for 15 seconds, at 14.000xg. Flow-through and collection tube were discarded. With the QIAamp spin column in a new 2 Ml collection tube, a volume of 700 µL of Buffer RW1 was added and a new centrifugation

at 14.000xg, for 15 seconds, was performed. Flow-though was once again discarded and the column placed on a new collection tube. A volume of 500 µL of Buffer RPE was pipetted to the column, followed by a centrifugation of 15 seconds, at 14.000xg. After changing the collection tube, the last step was repeated, and centrifuged at 16.000xg for 3 minutes. For the elimination of any residual Buffer RPE, the QIAamp spin column was transferred to one last collection tube and centrifuged at full speed, for 1 minute. Finally, the spin column was placed on a 1.5 Ml microcentrifuge tube and 50 µL of Rnase-free water were used to elute the RNA retained on the column's membrane.

The tubes with purified RNA were stored under a temperature of – 80 ºC until further manipulation.

(QIAamp RNA Blood Mini Handbook 2021).

3.4. RNA conversion into Cdna

This step was only performed on the samples destined to be analyzed on the Cetacean coronavirus survey (2.3.2).

The Cdna was reverse transcribed using Roche – Transcriptor High Fidelity Cdna Synthesis Kit, according to the manufacturer's instructions for the Reverse Transcription using random hexamer priming. (Transcriptor High Fidelity Cdna Synthesis Kit Handbook, 2017).

The protocol included two major steps. Firstly, in a 0.2 Ml microtube, a volume of 10 µL of purified RNA (sample) was mixed with 2 µL of pdN6 (random hexamer primer). The mixture was then subjected to a temperature of 65 ºC, in a 26hermos cycler with a heated lid, during 10 minutes. Immediately after this step, the tube was cooled on ice. This first step assures the annealing of the pdN6 to the RNA strand.

In the second step of the protocol, 4 µL of Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer (5X conc.), 0.5 µL of Protector Rnase Inhibitor (40 U/Ml), 2 µL of Deoxynucleotide Mix (10 Mm each), 1 µL of DTT and 1.1 µL of Transcriptor High Fidelity Reverse Transcriptase, were added. The tube was placed on a thermocycler and underwent the following temperatures: 29 ºC for 10 minutes, 48 ºC for 60 minutes and a final temperature of 85 ºC for 5 minutes, for inactivation of the reverse transcriptase. The Cdna was stored at -20 ºC until further manipulation.

3.5. RNA and DNA quantification and purity assessment

DNA and RNA's quality and concentration were measured using the Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer. This equipment allows the determination of DNA/RNA nanograms per microliter (ng/ µL).

The system is based on the intrinsic absorbance properties of DNA and RNA's nucleic acids, which absorption spectrum shows a characteristic peak at the 260 nm. Spectrophotometers measure the attenuation on the light that reaches the detector, after passing through a sample. The correlation of this values with the previously incident light is expressed as absorbance values, which provide the nucleic acid quantification. (T123 – Technical Bulletin NanoDrop Lite Interpretation of Nucleic Acid 260/280 Ratios 2012).

Sample's purity is confirmed by a A260/280 ratio (absorbance at 260 nm / absorbance at 280 nm) of 1.8-2 (Wilfinger et al. 1997).

The DNA concentrations (ng/µL) varied between 1.1 – 194.3 ng/µL (mean= 25.9 ng/µL) and the RNA concentrations (ng/µL) between 26.7 – 343.3 ng/µL (mean= 142,7 ng/µL). Table can be consulted on Annexes (Table 16).

3.6. Conventional Polymerase Chain Reaction (Cpcr)

The following table (Table 7) presents a summary of the primers used in both surveys performed on the present study.

Table 7. PCR assays performed in this study. Primer's sequences and the molecular weight of each amplicon (base pairs)

	Target Gene	Primer identification	Primer sequence (5´- 3')	Amplicon size	Reference
Poxvirus	DNApol	PoxFor	CAR GAA ATM AAA AAG AAR TTT CCA TC	150 bp	Sacristán et al. 2018
		PoxRev	ACG TTC TGT TAA RAA YCG TCT TAG TA		
	DNApol	Forward (PF-DNApol)	ATA CAG AGC TAG TAC ITT AAT AAA AG	543 bp	Bracht et al. 2006
		Reverse (PR-DNApol)	CTA TTT TTA AAT CCC ATT AAA CC		
Coronavirus	RNApol	Forward Pan_CoV_F1 Pan_CoV_F-2 Pan_CoV_F-3	GGT GGG AYT AYC CHA ART GYG A GAY TAY CCH AAR TGT GAY AGA GAY TAY CCH AAR TGT GAY MGH	440 bp 430 bp	Holbrook et al. 2021
		Reverse Pan_CoV_R-1 Pan_CoV_R-2	CCR TCA TCA GAH ARW ATC AT CCR TCA TCA CTH ARW ATC AT		

Primers described by Sacristán et al. and Holbrook et al. were degenerate primers. The presence of letters other than T, C, G and A, identifies a position where different nucleotides can be allocated (W: A/T; S: C/G; M: A/C; K: G/T; R: A/G; Y: C/T; B: C/G/T; D: A/G/T; H: A/C/T; V: A/C/G; N: A/C/G/T).

3.6.1. Cetacean poxvirus survey

Samples were analyzed through conventional PCR, using the VWR® Doppio thermocycler. A set of degenerate primers previously described in Sacristán et al., 2018 were chosen: Primer forward (PF): 5′- CAR GAA ATM AAA AAG AAR TTT CCA TC -3′, and Primer reverse (PR): 5′-ACG TTC TGT TAA RAA YCG TCT TAG TA -3′. The total PCR reaction volume was 25 µL containing: 12,5 µL of DreamTaq Green PCR Master Mix 2X (ThermoScientific), 25 pmol of each forward and reverse primer, 7.5 µL of Milli-Q® water and 50-100 ng of template DNA. The PCR amplification protocol was initiated with a denaturation step at of 94°C for 5 minutes; followed by 40 cycles at 94°C, 15 seconds, annealing at 50°C, 30 seconds and extension at 72°C, 30 seconds; and a final extension step at 72°C, 7 minutes. Positive samples resulted on the amplification of a product with 150 base pairs (bp).

Positive samples were further tested for the DNA polymerase gene, using the conventional PCR protocol described in (Bracht et al. 2006). A set of two primers was used: Primer forward (FP-DNApol): 5'-ATA CAG AGC TAG TAC ITT AAT AAA AG-3; and Primer reverse (RP-DNApol): 5'-CTA TTT TTA AAT CCC ATT AAA CC-3'. The reaction volume of 25 µL included: 12.5 µL of AccuStart II PCR ToughMix 2X (Quanta), 2.5 µL of Primer forward (25 mol/µL), 2.5 µL of Primer reverse (25 mol/µL), 4.5 µL of Milli-Q® water and 50-100 ng of template DNA. Amplification conditions included an initial denaturation at 94°C for 1 minute, followed by 39 cycles of denaturation at 94°C for 30 seconds, an annealing step at 45°C for 30 seconds, and an elongation step at 72°C for 30 seconds, ending with a final extension step of 72°C for 10 minutes. Positive samples resulted on a product with the molecular weight of 543 base pairs (bp).

3.6.1.1. Sensitivity of the PCR system (Limit of detection)

In order to infer the sensibility of the system described by Sacristán et al, 10-fold serial dilutions of the positive control were put to test. Template dilutions, starting on 10^{-4} until 10^{-9}, underwent the PCR assay in the same conditions as previously mentioned (2.6.1.). The highest dilution where amplification occurred, was considered the LOD (Limit of Detection).

Mathematically, sensibility is defined by the ratio between the true positive tests (TP) and the real positive (RP). In its turn, the real positive tests (RP) are the sum of the true positive (TP) testes with the false negative (FN). Once the acquisition of reference materials from positive cetacean poxvirus was not possible, the FN were not deducted. The LOD value was used as a sensibility parameter.

3.6.1.2. Specificity of the PCR system

In order to infer the specificity of the system, two (n=2) random samples, belonging to organs from cetaceans previously proven positive to virus other than poxvirus, were tested, under the same amplification conditions described by Sacristán et al.

Additionally, positive samples (n=4) chosen randomly, were cloned, recombinant plasmids were produced and sent to STAB VIDA (Genetics Laboratory), for Sanger sequencing.

3.6.2. *Cetacean coronavirus* survey

Samples were analyzed using a semi nested Pan-Coronavirus conventional PCR assay, as described in Holbrook et al. 2021.

The first PCR resorted to a set of three primers: Primer forward 1 (Pan_CoV_F-1): 5'-GGT GGG AYT AYC CHA ART GYG A-3', Primer reverse 1 (Pan_CoV_R-1): 5'-CCR TCA TCA GAH ARW ATC AT-3', and Primer reverse 2 (Pan_CoV_R-2): 5'-CCR TCA TCA CTH ARW ATC AT-3'. The reaction volume of 25 µL included: 12.5 µL of DreamTaq Green PCR Master Mix (2X), 50 pmol of each Primer (forward 1; reverse 1; reverse 2), 5 µL of Milli-Q® water and 1.5 µL of cDNA template (the template volume was adjusted in each sample, in order to have a cDNA concentration between 50-100 ng). The amplification conditions applied were: initial denaturation step at 94 °C for 5 minutes, followed by 25 cycles at 94 °C for 30 seconds, annealing at 48 °C for 30 seconds, and 72 °C for 1 minute; and finally, an extension step at 72°C for 5 minutes. The resulting product of this first PCR had 440 base pairs (bp).

The second amplification used the same reverse primers as the previous amplification and two new forward primers: Primer forward 2 (Pan_CoV_F-2): 5'-GAY TAY CCH AAR TGT GAY AGA-3' and Primer forward 3 (Pan_CoV_F-3) 5'-GAY TAY CCH AAR TGT GAY MGH-3'. The reaction volume of 25 µL included: 12.5 µL of DreamTaq Green PCR Master Mix (2X), 50 pmol of each primer (forward 2; forward 3; reverse 1; reverse 2), 2 µL of Milli-Q® water and 2.5 µL from the previous PCR reaction. The followed PCR conditions were: an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an elongation step at 72°C for 1 minute, ending with a final extension step of 72°C for 5 minutes.

The final product of this semi-nested PCR had a molecular weight of 430 base pairs (bp).

3.6.2.1. System Validation

Being a PAN system, the validation was performed with samples from the 3 different coronavirus genera. For this purpose, several materials, previously known to be coronavirus positive, were tested: canine feces, for Canine coronavirus (Alphacoronavirus); feline feces, for Feline coronavirus (Alphacoronavirus); goat field isolate, for Bovine coronavirus (Betacoronavirus); Gallivac IB88 (strain CR88121) vaccine, for avian Infectious Bronquitis (Gammacoronavirus); and Biovac IB (H120 strain), for avian Infectious Bronquitis (Gammacoronavirus).

Total RNA was extracted using the Qiamp RNA blood minikit, according to the manufacturer's instructions (2.3. DNA and RNA extraction). After PCR testing, fragments were purified, recombinant plasmids were produced and sequenced.

3.7. Electrophoresis in Agarose gel

All PCR products resultant from both surveys were visualized by horizontal electrophoresis in agarose gel. The agarose gels were prepared at 1.5% in TE Buffer (Tris-EDTA). While preparing the gel, a staining agent was incorporated to allow the nucleic acids' visualization under UV light. In this case, GelRed® Nucleic Acid Stain was used.

To infer the bands' a molecular size marker NZYDNA Ladder V (Nzytech) was loaded in parallel with the samples.

The runs were performed under a voltage of 120V, for a medium period of 45 minutes, and the results were visualized through the Bio-Rad's Gel Doc XR+ system and documented.

3.8. Bands purification from agarose gel

When bands with the expected molecular weight were present in the agarose gel (visualization under UV light), their extraction and purification was performed.

For this purpose, the Zymoclean Gel DNA Recovery Kit was used. The desired DNA fragments (bands) were cut, with a scalpel blade, and placed on a 2 mL microcentrifuge tube. Agarose Dissolving Buffer (ADB) was added to the same microcentrifuge tube, in a 3:1 proportion (3 volumes of ADB to each volume of excised agarose gel). This step was followed by an incubation at 42 °C for 10 minutes. After complete dissolution, que melted agarose gel was transferred to a Zymo-Spin™ Column in a collection tube. The column was centrifuged at 14.000xg for 1 minute and the resulting flow-through discarded. Two consecutive washes with 200 µL of DNA Wash Buffer were performed, each one followed by a quick centrifugation step of 30 seconds, at 14.000xg. Finally,

placing the Zymo-Spin™ Column on a 1.5mL microcentrifuge tube, DNA was eluted with 10 µL of DNA Elution Buffer. A final centrifugation was performed at 14.000xg for one minute.
(Instruction Manual – Zymoclean™ Gel DNA Recovery Kit – Zymo Research).

The purified fragments were quantified using the Thermo Scientific™ NanoDrop™ and stored at -20 ºC until further manipulation.

3.9. Plasmid Cloning

Cloning of PCR products was performed for laboratory convenience such as sequencing of low molecular weight amplicons and production of plasmid recombinants for establishment of positive controls.

3.9.1. Blunting reaction (Ligation)

On a 1.5 mL microtube, several components were added with the following volumes: 10 µL of Reaction Buffer (2X), 2 µL of Purified PCR product (the insert's volume was individually adjusted in order to obtain optimal efficiency in the ligation reaction – Table 8), 5 µL of nuclease-free Water and 1 µL of DNA Blunting Enzyme.

The mixture was incubated at 70 °C for 5 minutes and immediately cooled on ice. Maintaining the microtube on ice, two final components were pipetted into the mixture: 1 µL of pJET1.2/blunt Cloning Vector (50 ng/µL) and 1 µL of T4 DNA Ligase. After vortexing, the final product (Ligation) was left at room temperature for 5 min.

Table 8. Length of PCR product in base pairs and correspondent advised quantity to use on the ligation reaction. Source: Product Information Thermo Scientific CloneJET PCR Cloning Kit 2012

LENGTH OF PCR PRODUCT (BP)	OPTIMAL PCR PRODUCT QUANTITY FOR LIGATION REACTION, (0.15 PMOL ENDS)
100	5 ng
300	15 ng
500	25 ng
1000	50 ng
2000	100 ng
3000	150 ng
4000	200 ng
5000	250 ng

The pJET1.2/blunt (Figure 9) is a linearized cloning plasmid. After the ligation reaction, the vector recircularizes, with or without the insert. When the vector recircularizes with the desired insert, a lethal restricition enzyme encompassing the

plasmid cloning site is interrupted allowing the growth of the recombinant bacteria. If the vector recirculizes whithout the insert the letal gene is expressed, thus preventing bacterial growth. Only the recombinant clones, with incorporated insert are viable. Screening is then performed to confirm the specificity of the inserted fragment.
(Product Information Thermo Scientific CloneJET PCR Cloning Kit 2012)

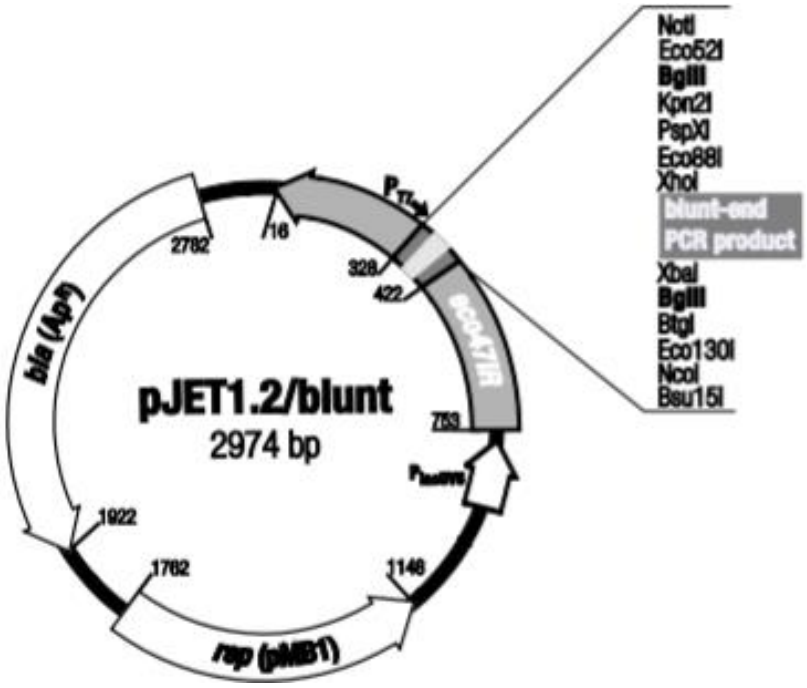


Figure 9. Representation of the pJET1.2/blunt circularized plasmid with the intended insert. Source: Product Information Thermo Scientific CloneJET PCR Cloning Kit 2012

3.9.2. Competent cells transformation

This step of the cloning process started by unfreezing a 1.5 mL vial containing 25 µL of E. coli DH5-α (competent cells), on ice. When fully melted, 2 µL of the Ligation mixture were added to the competent cells. The mixture was subjected to a thermal shock starting with ice for 20 minutes, followed by 45 seconds at 42 °C (dry-block) and finally, ice for 10 minutes. The cells were then transferred to a 50 mL falcon tube, containing 1 mL of S.O.C. (Super Optimal broth) medium, and incubated in an orbital incubator for 90 minutes, at 37 °C/ 180 rpm. After this incubation period, the medium was centrifuged at 1.500xg for 1 min, and the supernatant discarded by carefully inverting the tube. The pelleted cells were carefully resuspended in the remaining medium, and cultivated in a LB (Luria Bertani) agar plaque, containing 100µg/mL of ampicillin. Plates were incubated at 37°C, overnight.

3.9.3. Culture screening

Well isolated, homogeneous colonies were chosen from the LB agar/ampiciline plates (prepared on the previous step). Each individual bacteria colony was ressupended in a 1.5 mL microtube, containing 50 µL of PBS, and used directly as template to infer the presence of the desired insert on the E. coli DH5-α (competent cells). The PCR screening used as forward and reverse primers specific to pJET1.2: Primer forward (PFpJET1.2): 5'-CGACTCACTATAGGGAGAGCGGC-3'; Primer reverse (PRpJET1.2): 5'-AAGAACATCGATTTTCCATGGCAG-3'. A PCR reaction mixture was prepared containing: 12.5 µL of DreamTaq Green PCR Master Mix (2X), 1 µL of Primer reverse (25 pmol/µL), 1 µL of Primer forward (25 pmol/µL), 8 µL of Rnase-free water and 2,5 µL of template.

The PCR products were visualized on an 1.5% agarose gel. Products with the expected molecular weight confirmed the construction of the specific recombinant plasmid.

3.9.4. Extraction and purification of recombinant DNA (minipreps)

Positive colonies were transferred into 10 mL of liquid LB medium with ampicillin and incubated overnight at 37 °C/180 rpm in an orbital incubator.

For the plasmid DNA extraction, the Thermo Scientific™ GeneJET Plasmid Miniprep Kit was used. The bacterial cultures were centrifuged for 10 minutes, at 1.500xg. The supernatant was discarded and the pelleted cells were carefully resuspended, in 250 µL of Resuspension Solution. Cells' suspension was transferred to a new 1.5 mL microcentrifuge tube and 250 µL of Lysis Solution was added and mixed, slowly inverting the tube until bacterial lysis. The lysing solution ensures the rupture of the cells' wall. Reaction was therefore neutralized through the addition of 350 µL of Neutralization Solution, and slowly mixed by inverting the tube 5 times. The adding of the Neutralization Solution induces the precipitation of high molecular weight DNA (genomic DNA) but not of low molecular weight DNA (plasmid DNA). A centrifugation step was performed at 16.000xg for 5 minutes, and the resultant supernatant was decanted to the GeneJET spin column in a collection tube. Pelleted cells debris and high molecular weight genomic DNA were discarded. The solution was once again centrifuged at 16.000xg for 1 minute and the flow through discarded. The immobilized plasmid DNA on the GeneJET spin column's membrane, was then subjected to three washing steps, each with 500 µL of Wash Solution at 16.000xg for 1 minute. Flowthrough from each wash was discarded. The plasmid DNA was finally eluted by adding 50 µL of the Elution Buffer followed by a centrifugation, at 16.000xg for 1 minute and stored at -20 ºC until further manipulation.

(Product Information Thermo Scientific CloneJET PCR Cloning Kit 2012)

3.10. Sanger Sequencing

All amplicons with the expected molecular weight, including purified PCR reactions amplified from the different samples and targeting poxvirus or coronavirus, and recombinant plasmid DNA were subjected to nucleotide sequencing. All samples were sequenced in both senses, using the specific forward and reverse primers of each PCR reaction.

3.10.1. Cycle sequencing reaction

For these purposes, the ABI Prism BigDye Terminator v3.1 Cycle sequencing kit was used. The initial amplification reaction was carried out by mixing 2 µL of Ready Reaction Premix (2.5X), 1 µL of BigDye Sequencing Buffer (5X), 12.5 pmol of specific Primer, template DNA (volume according to the amplicon length/concentration (Table 9) and water to a final volume of 10 µL.

Table 9. Correlation between template length (base pairs) and recommended DNA quantity (ng) to use on the sequencing reaction. Adapted from: BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol, Applied Byosystems

Template	DNA concentration
PCR product:	
100–200 bp	1–3 ng
200–500 bp	3–10 ng
500–1000 bp	5–20 ng
1000–2000 bp	10–40 ng
>2000 bp	20–50 ng

The amplification reaction included an initial denaturation step at 96 ºC, for 1 minute; followed by 25 cycles of denaturation at 96 ºC for 10 seconds; hybridization at the primer annealing temperature for 5 seconds; and 60 ºC for 30seg -1 min.
(BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol, Applied Byosystems 2002)

3.10.2. DNA Precipitation

Following the previous reaction, the DNA was precipitated with 2µL of 125mM EDTA, 2µL of 3M Sodium Acetate (NaAc) and 50µL of Ethanol (EtOH) 95%. The mixture was refrigerated at -20ºC for at least 30 minutes and centrifuged for 20 minutes, at 16.000xg, in a refrigerated centrifuge at 4ºC. The supernatant was carefully removed

and 170µL of Ethanol (EtOH) 70% were added followed by an additional centrifugation under the previous conditions for 10 minutes. Supernatant was carefully discarded; the DNA pellet was dried at 65 ºC for 15 minutes and resuspended in 20µL of formamide.

Samples were analyzed on an Applied Biosystems – 3130 Genetic Analyzer.

3.11. Phylogenetic analysis.

The nucleotide sequences obtained through Sanger sequencing, were subjected to the BLAST – Basic Local Alignment Search Tool (National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), a program that finds regions of similarity between nucleotide or amino-acidic sequences, basing this comparison on the GenBank database of sequenced and identified sequences. With this program we can also infer the percentage of similarity/identity and the GeneBank accession number of each retrieved sequence with a significant identity with our query. The significance of the identity is related with the E value. This parameter describes the probability of random background sequences. The lower the E value stands, the higher the significance of the retrieved sequences.

3.11.1. Cetacean poxvirus survey

The phylogenetic analysis performed on the Cetacean poxvirus positive samples was based on the partial DNA polymerase gene.

To build the phylogenetic tree, representative members of the Poxviridae family were chosen based on their Genebank Acession number, namely: Vaccinia virus (YP232947), Pseudo cow Poxvirus (YP003457330), Bovine popular stomatitis Poxvirus (AKC03451), Swine Poxvirus (NP570196), Yaba monkey Poxvirus (NP938295), Camel Poxvirus (NP570451), Nile crocodile Poxvirus (YP784249) and Turkey Poxvirus (YP009177089). Different Cetacean poxviruses, assigned to each known subgroup (CePV1-6) were chosen: CePV-1 (DQ071860), CePV-2 (AY846759), CePV-3 (AY463006), CePV-4 (KC409036), CePV-5 (JN654445) and CePV-6 (JN654442). An unassigned Cetacean poxvirus was also included (KC409037) (Table 10).

The analysis involved the 26 amino acid cropped sequences and all positions with missing data were eliminated. The evolutionary proximity was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). The bootstrap values are shown next to each branch. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 3.1155)). The evolutionary analyses were conducted in MEGA7 (Felsenstein 1985).

Table 10. Viral genome used in the construction of the phylogenetic tree and respective GenBank accession number.

Virus	GenBank accession number
Cetacean poxvirus (*Tursiops aduncus*)	AY463006
Cetacean poxvirus (*Stenella coeruleoalba*)	KC409037
Cetacean poxvirus (*Stenella coeruleoalba*)	JN654445
Cetacean poxvirus (*Phocoena phocoena*)	KC409036
Cetacean poxvirus (*Delphinus delphis*)	JN654442
Cetacean poxvirus (*Stenella coeruleoalba*)	DQ071860
Cetacean poxvirus (*Balaena mysticetus*)	AY846759
Turkey pox	YP009177089
Nile crocodilepox virus	YP784249
Vaccinia virus	YP232947
Camelpox virus	NP570451
Yaba monkey tumor virus	NP938295
Swinepox virus	NP570196
Bovine papular stomatitis virus	AKC03451
Pseudocowpox virus	YP003457330

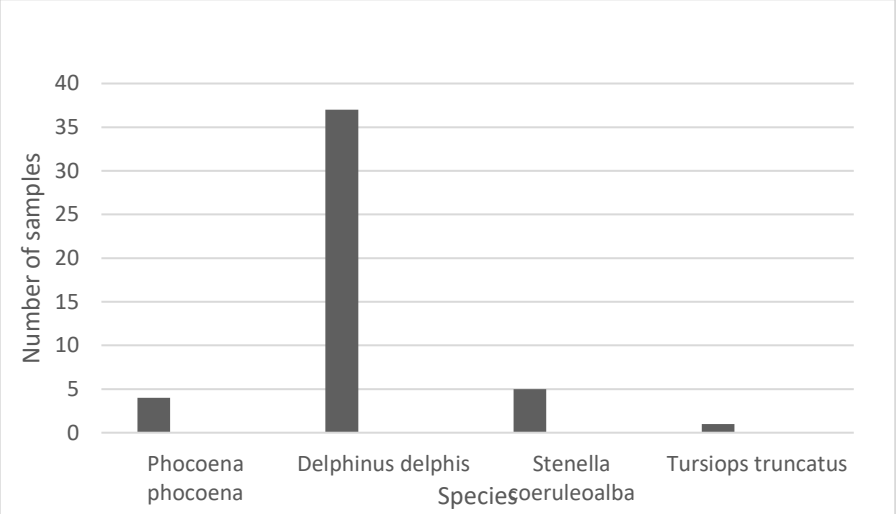
4. Results

4.1. Sample characterization

4.1.1. Cetacean Poxvirus survey

A total of forty-seven (n=47) samples were analyzed, including 4 different species of dolphins (*Delphinus delphis*, *Tursiops truncates*, *Phocoena phocoena* and *Stenella coeruleoalba*) (Graphic 1).

Graphic 1. Species distribution in the Cetacean poxvirus survey.



Data related to each individual (date of necropsy, local of the stranding, type of stranding, decomposition state, approximate age, body condition score, sex, attributed cause of death and macroscopic observations) was only requested to animals which further on tested positive and were able to be sequenced.

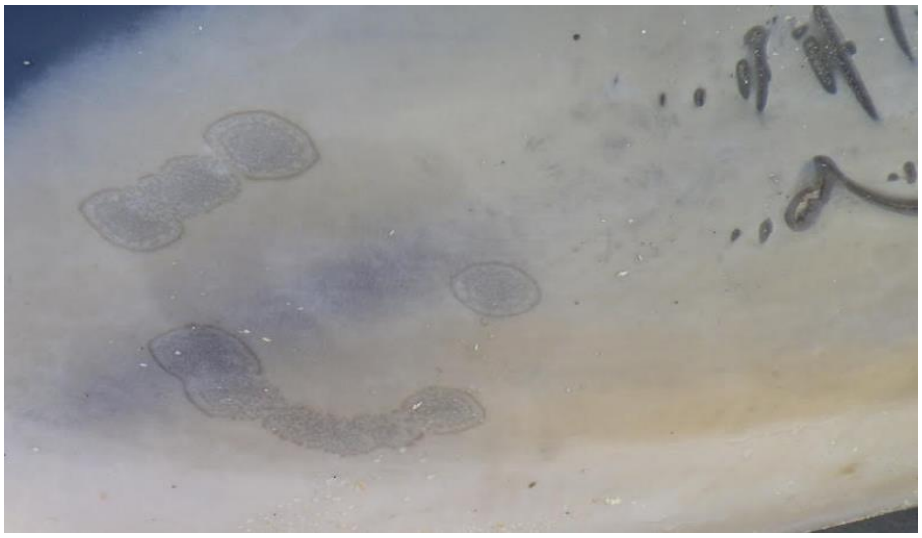


Figure 10. Skin lesion collected from a common dolphin (*Delphinus delphis*). Picture given by the North Marine Mammal Stranding Network.

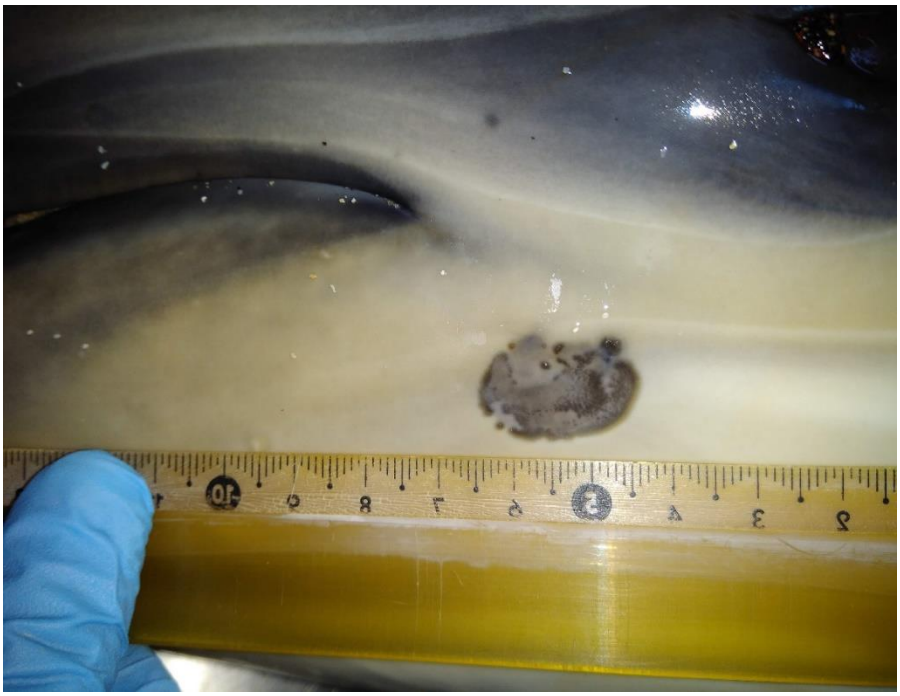
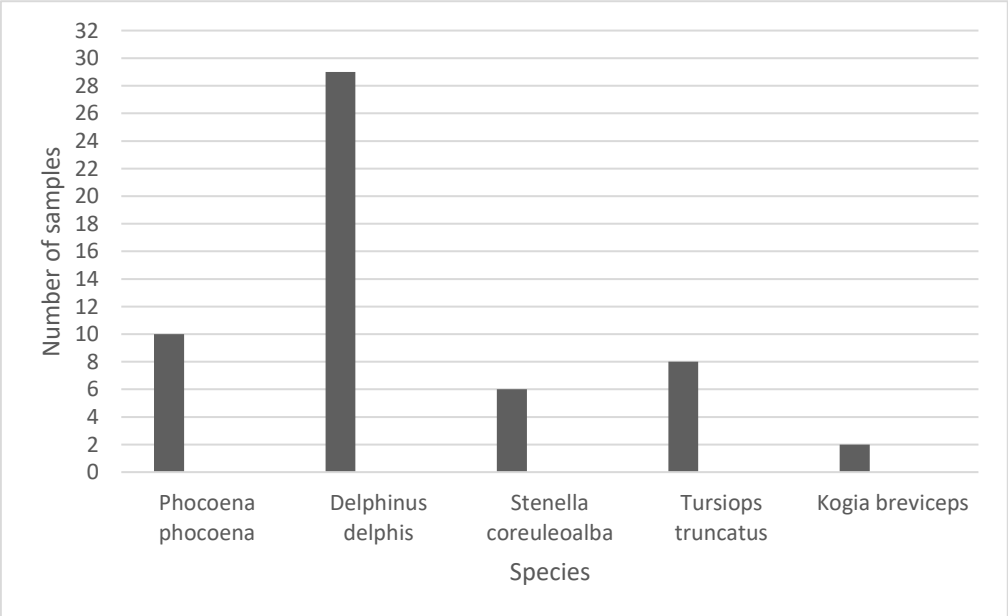


Figure 11. Skin lesion collected from a common dolphin (*Delphinus delphis*). All suspect lesions are photo documented. Picture given by the North Marine Mammal Stranding Network.

4.1.2. Cetacean Coronavirus survey

A total of fifty-five (n=55) samples were analyzed, including samples from 5 cetacean species (*Delphinus delphis*, *Tursiops truncates*, *Phocoena phocoena, Stenella coeruleoalba and Kogia breviceps*) (Graphic 2).

Graphic 2. Species distribution in the Cetacean coronavirus survey.



4.2. Analysis of PCR products

4.2.1. Cetacean poxvirus survey

The sensibility of the assay described by Sacristán et al. was assessed testing a sample from a *Tursiops truncates'* skin sample, previously characterized as positive (Figure 12). The limit of detection achieved by the assay was considered to be the dilution of 10^{-9}, showing strong amplification bands in all dilutions. This makes the present system a useful survey tool, delivering results with high sensibility.

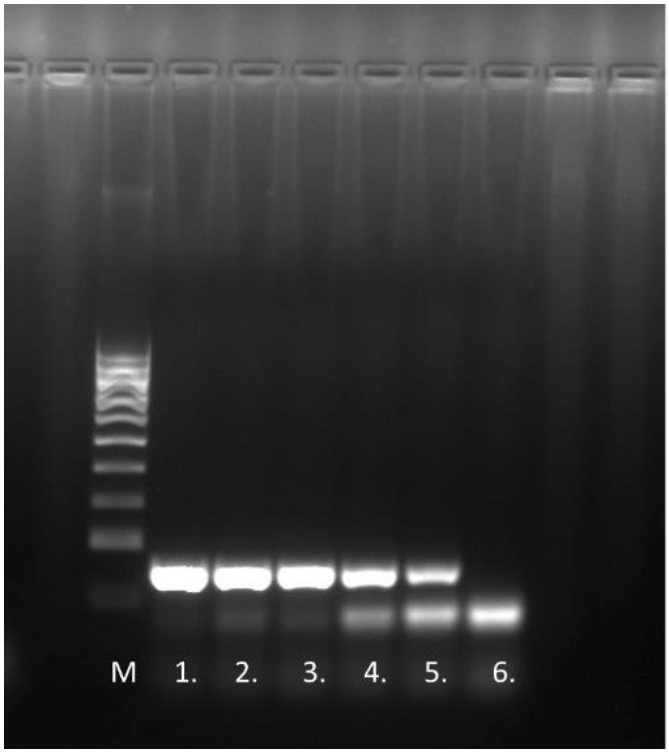


Figure 12. Sensibility test performed with primers described by Sacristã et al. Template used on this reaction was the positive control. (M) Molecular weight marker; (1) 10^{-5}; (2) 10^{-6}; (3) 10^{-7}; (4) 10^{-8}; (5) 10^{-9}; (6) Negative control. Original

Following sensitivity assessment, the molecular screening was performed in the samples' previously extracted DNA, yielding fragments of 150 bp (Figure 13). Twenty-nine (n=29) samples were considered positive (29/47; 61.7%).

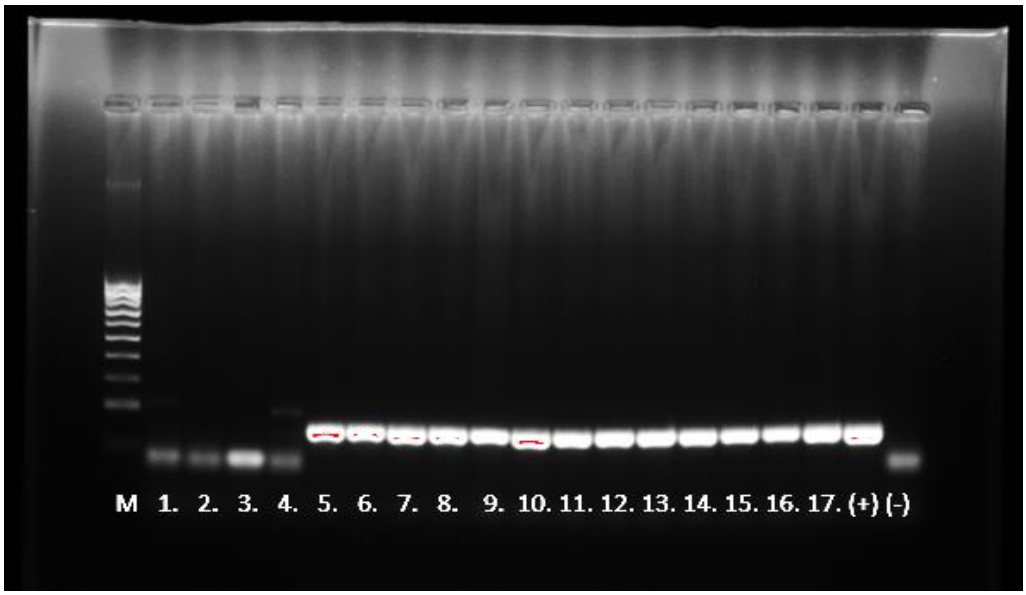
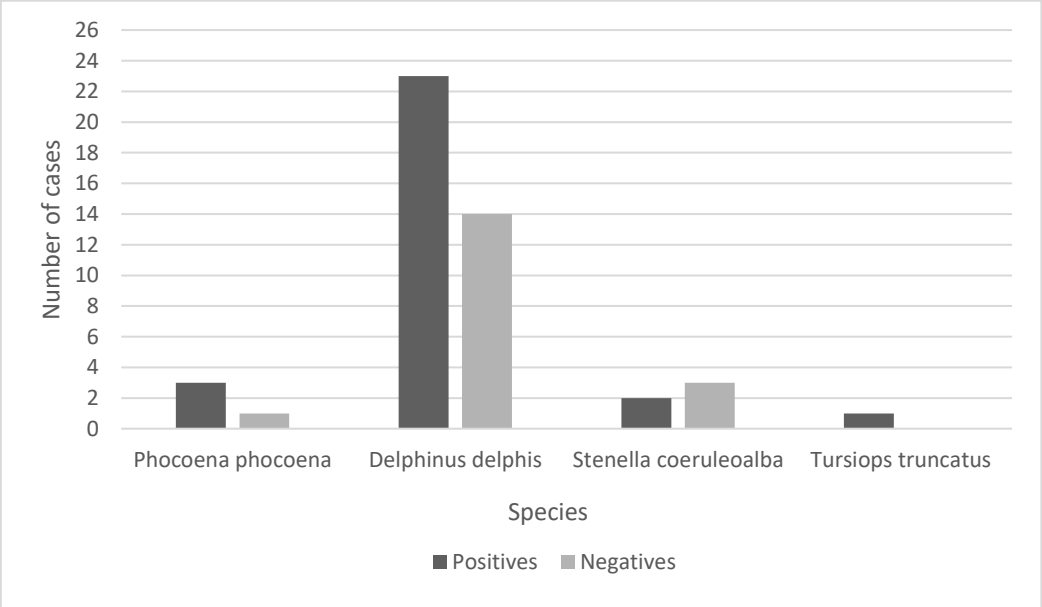


Figure 13. PCR amplification, using the primers described by Sacristán et.al. (M) Molecular weight marker; (1-17) Samples from *Delphinus delphis*; (+) Positive control; (-) Negative control. Original

A percentage of 85% of the *Phocoena phocoena* (3/4), 62,16% of the *Delphinus delphis* (23/37), 40% of the *Stenella coeruleoalba* (2/5) and 100% of the *Tursiops truncates* (1/1) tested positive to the molecular assay (Graphic 3).

Graphic 3. Distribution of the results obtained through the Cetacean poxvirus survey using the PCR assay described by Sacristán et al. Results organized by species



To confirm the amplification specificity of the assay described by Sacristán et al., four (n=4) [DD160/14, DD161/14, DD308/14 and DD240/14] PCR products, were cloned, sequenced (STAB VIDA, Portugal) and the nucleotide sequence submitted to the Basic

Local Alignment Search Tool – NCBI), revealing a 96% identity with Cetacean Poxvirus sequences with a significant lower E value (Figure 14).

Cetacean poxvirus 1 isolate C7 DNA polymerase gene, partial cds

Sequence ID: **MH005249.1** Length: 464 Number of Matches: 1

Range 1: 213 to 362

Score	Expect	Identities	Gaps	Strand	Frame
244 bits(132)	2e-60()	144/150(96%)	0/150(0%)	Plus/Plus	

```
Query  1    CAAGAAATCAAAAAGAAGTTTCCATCACCTAGATATATAGTTGTACATTGCGAGCCACGT  60
            || |||||| |||||||| ||||||||||||||||||||||||||||||| |||||||||
Sbjct  213  CAGGAAATAAAAAAGAAATTTCCATCACCTAGATATATAGTTGTACATTGTGAGCCACGT  272

Query  61   TTTAAGAATTTAATTTCTGAAATATCCATATTCGATAGAGAAGTTGAAGGCACTATACCT  120
            |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273  TTTAAGAATCTAATTTCTGAAATATCCATATTCGATAGAGAAGTTGAAGGCACTATACCT  332

Query  121  AGAATACTAAGACGATTTTTAACAGAACGT  150
            |||||||||||||||||| ||||||||||||
Sbjct  333  AGAATACTAAGACGATTCTTAACAGAACGT  362
```

Figure 14. Results from BLAST search from the sequence obtained through the subject DD308/14. The sequenced is aligned with MH005249.1 with a 96% of identity, 100% query cover and a 2e-60 E-value. Source: https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Positive samples (n=23) (Sacristán et al.), were further tested with the PCR assay described by Bracht et al., targeting the DNA polymerase gene and yeilding an amplicon of 543 bp. In this assay 19/23 samples were sucessfully amplified (82,6%) (Figure 15).

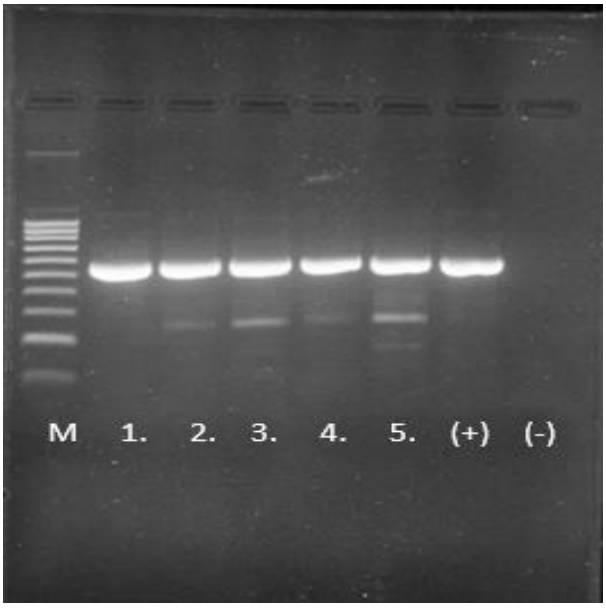
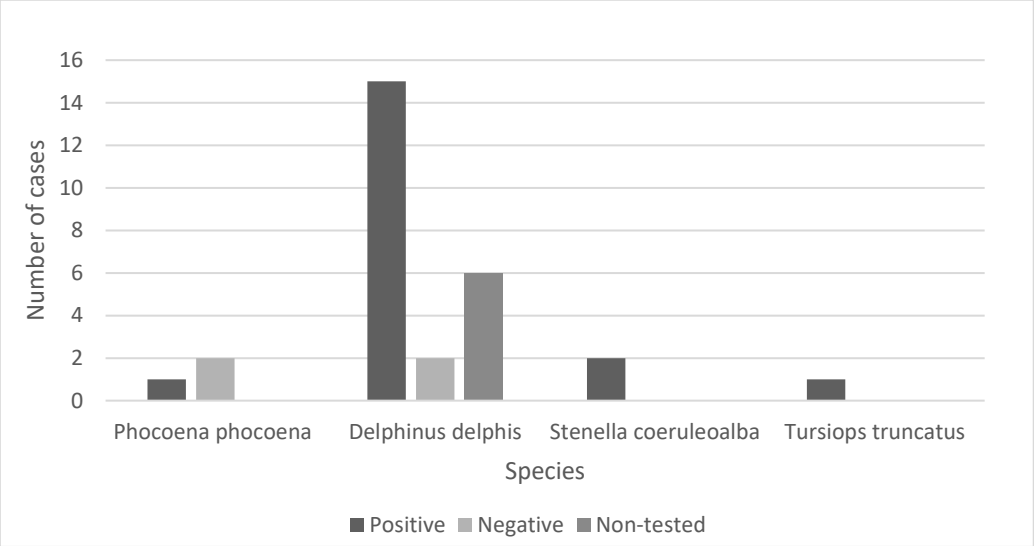


Figure 15. PCR amplification in agarose gel electrophoresis, using primers targeting the DNA polymerase gene. (M) Molecular weight marker; (1-5) *Delphinus delphis* samples; (+) Positive control; (-) Negative control. Original

The absence of successful amplification of the DNApol gene in four (n=4) samples may be related to low viral titre, since in the previous analysis they had already

shown a highly tenuous amplififcation band. However, all four different cetacean species included in the survey, remained represented amongst the positive samples (Graphic 4).

Graphic 4. Results obtained through the Cetacean poxvirus survey using the PCR assay described by Bracht et al. Distribution organized by species.



4.2.1.1. Sequencing

Concerning the amplicons targeting the DNA polymerase gene (Bracht et al. 2006) only twelve (12/19) were able to be sequenced by Sanger sequencing [DD251/14, DD159/14, DD160/14, DD162/14, DD165/14, DD180/14, TT233/12, DD237/14, DD156/14, DD262/14, DD292/14 and SC295/14 DD256 e DD152], and all were confirmed specific by BLAST analysis. For the phylogenetic analysis and given the lack of quality reads the nucleotide sequences of the remaining samples [DD240/14, DD249/14, DD263/14, DD279/14, SC280/14, DD289/14 and PP296/14] were not included.

Nucleotide sequences are presented on Annexes (Table 13).

4.2.1.2. Phylogenetic analysis

For the phylogenetic analysis, several Poxvirus sequences included in the poxviridae family, were chosen. Also sequences from each Cetacean poxvirus' clusters (CePV1-6), previously identified by Barnett et al. were included, to infeer the distribution pattern of the sequences of the present study (Table 10).

Due to the high nucleotide variability of Poxvirus sequences within the DNA polymerase, each sequence was translated and a multiple alignment of the amino-acidic sequences was performed (Figure 16.) for construction of the phylogenetic tree (Figure 17).

Figure 16. Alignment of amino-acidic sequences resultant from the PCR products obtained in the assay targeting the DNA polymerase gene, during the Cetacean poxvirus survey. Alignment was performed resorting to MEGA7 program. Original.

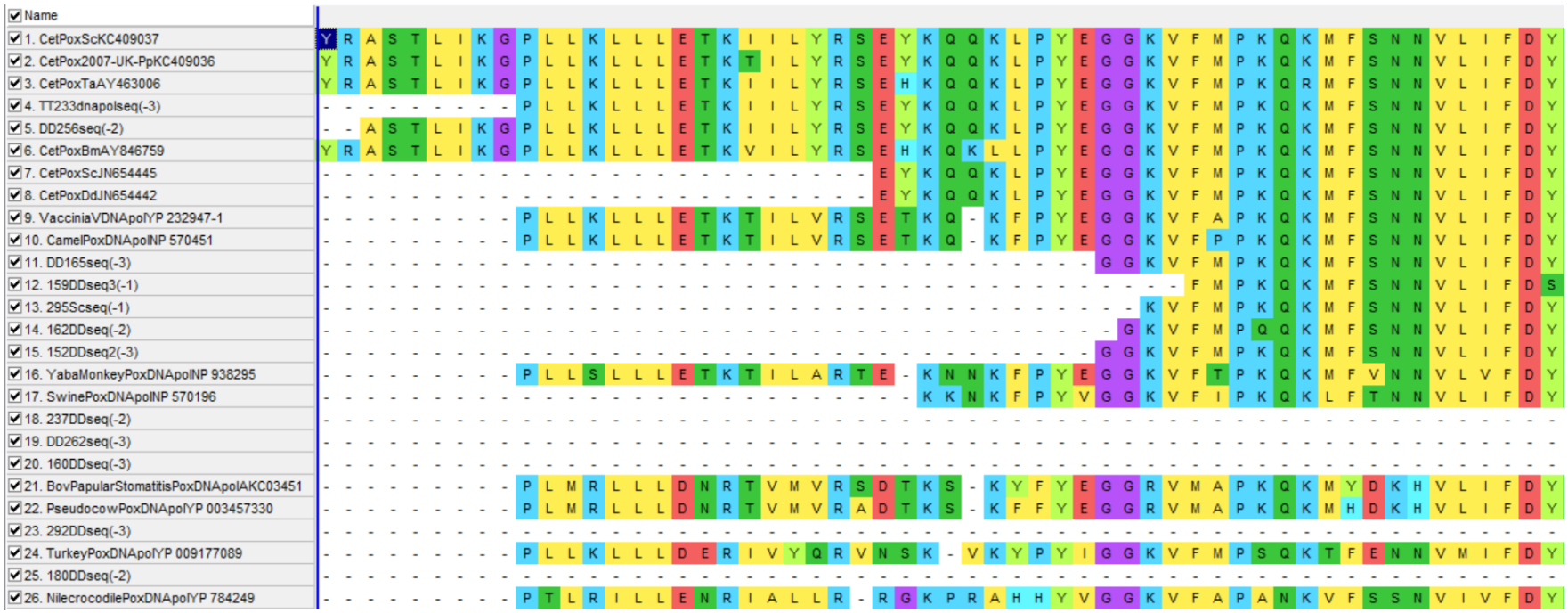


Figure 16. Continuation I.

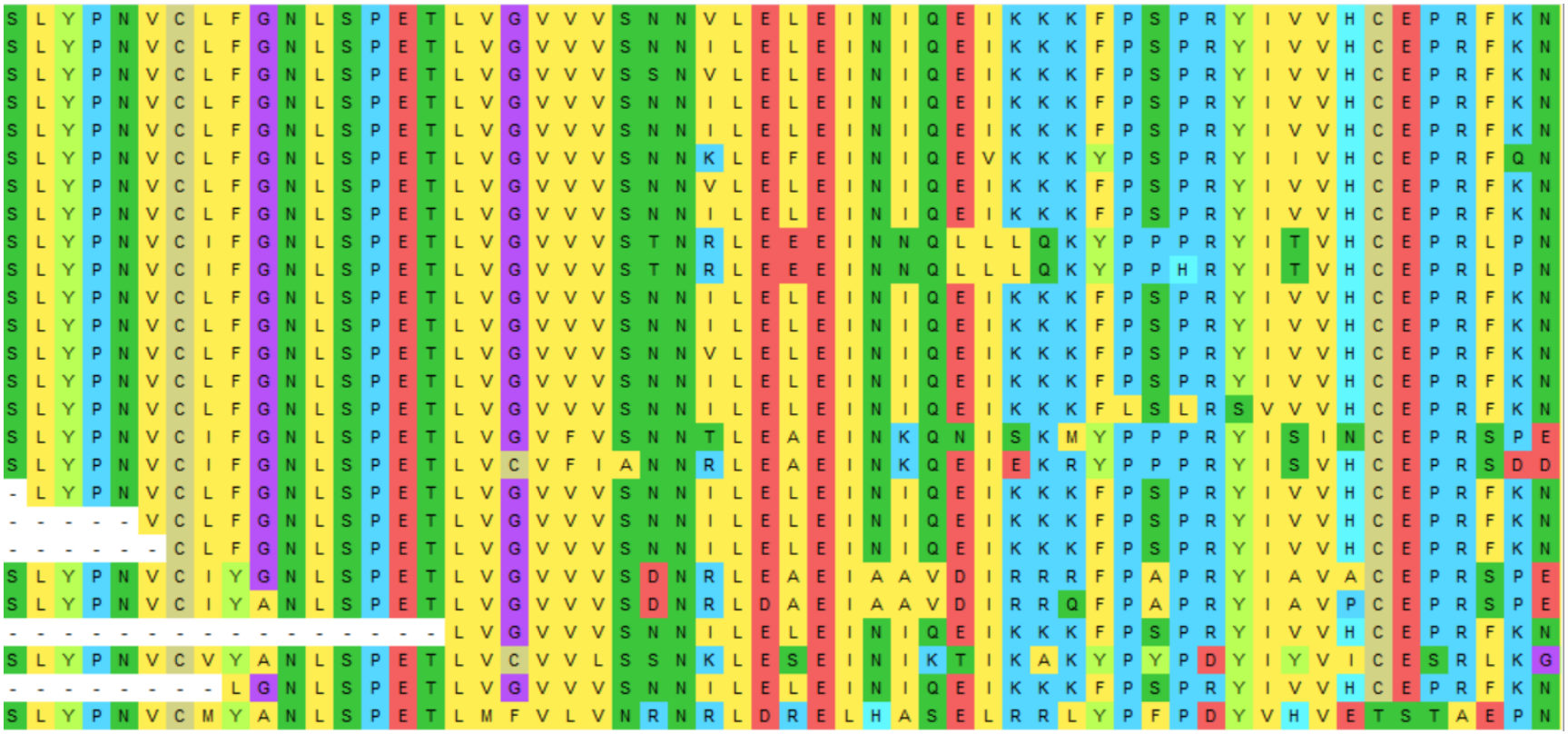
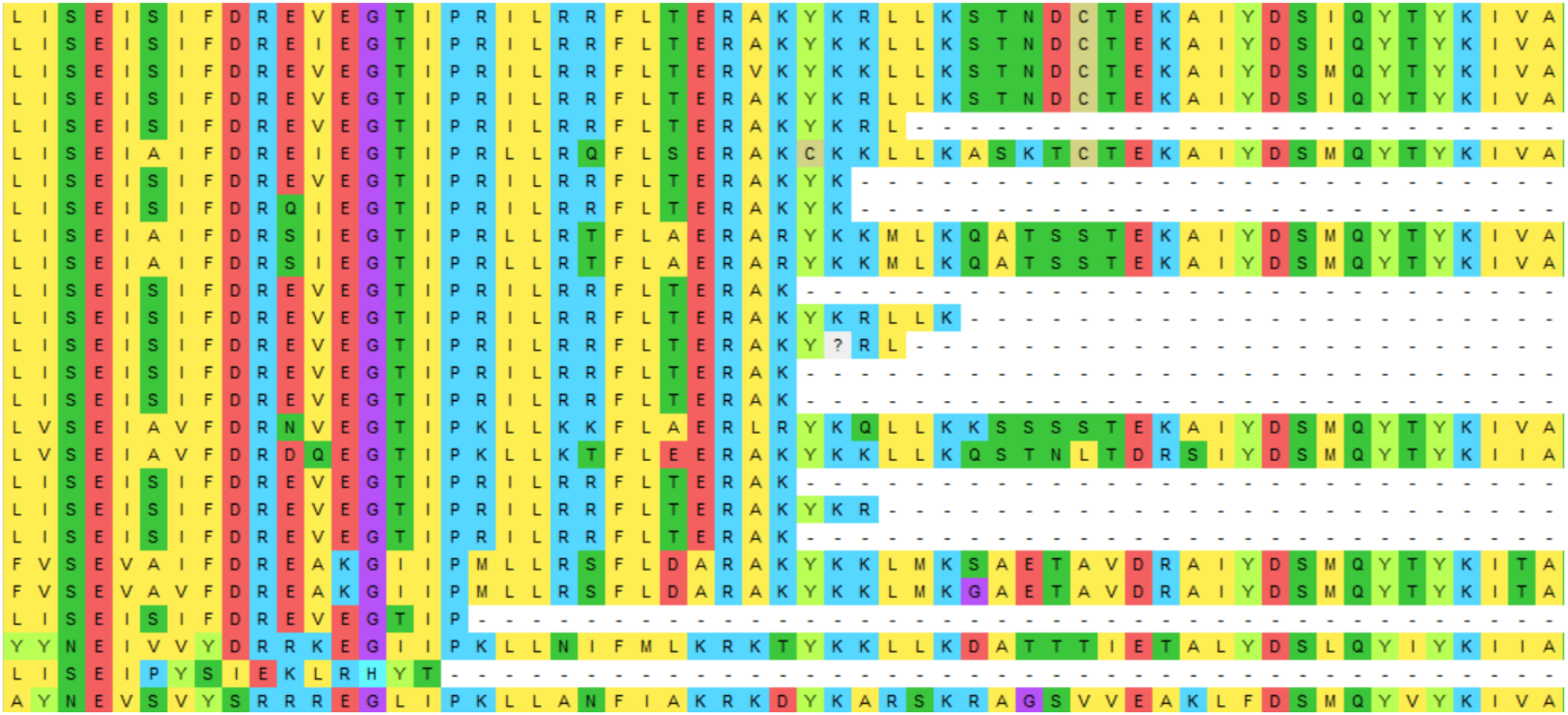


Figure 16. Continuation II.



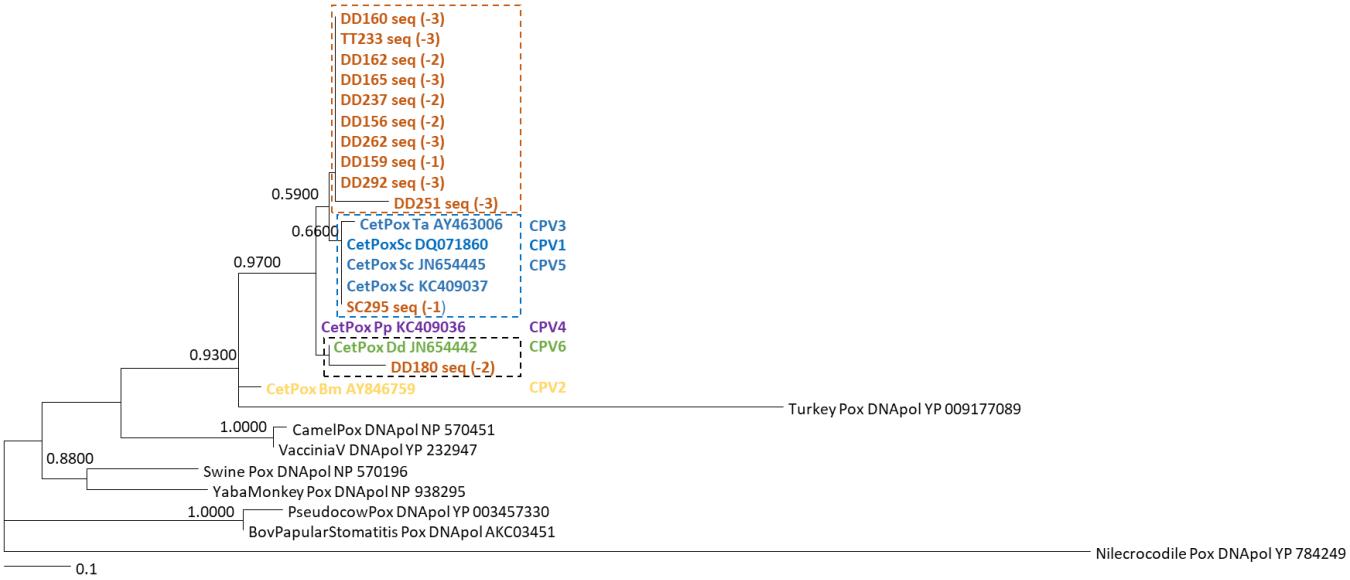


Figure 17. Phylogenetic tree obtained through the nucleotide alignment performed with samples gathered in the present study. The colour coding represents the samples from the present study (in orange: DD262/14, DD160/14, DD237/14, DD162/14, DD159/14, DD165/14, DD156/14, TT233/12, DD292/14, SC295/14, DD251/14, DD180/14). The phylogenetic tree was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). The tree topology was constructed by applying Neighbor-Join algorithms to a matrix of pairwise distances estimated using the JTT model. A Gamma distributed model was used to calculate variation in the evolution rate among sites. Gaps and missing data were removed from the alignment. The phylogenetic analysis was performed in MEGA7 software (Kumar et al. 2016)

The phylogenetic tree based of a partial segment of the DNA polymerase protein clearly resolves an independent branching grouping Cetacean Poxvirus (bootstrap 97%) although CePV-2 branch outliers from the remaining groups. Within Cetacean poxvirus, CePV-1, 3 and 5 cluster together with sequence SC295/14, supported by 66% of bootstrap value. Sequences DD180/14 cluster with CePV6, while the remaining samples [DD262/14, DD160/14, DD237/14, DD162/14, DD159/14, DD165/14, DD156/14, TT233/12, DD292/14, DD251/14] are united within a branch supported by 59% of bootstrap value.

Considering the phylogenetic distribution proposed by Barnett et al., the vast majority of the samples described in this work [DD262/14, DD160/14, DD237/14, DD162/14, DD159/14, DD165/14, DD156/14, TT233/12, DD292/14, DD251/14] may represent an additional subgroup within the cetacean poxvirus, due to their independent clustering within the tree.

4.2.1.3. Data analysis

Data concerning the individuals whose samples tested positive for Cetacean poxvirus was requested to the Stranding Network. Information concerning sex, approximate age, date of stranding, state of decomposition, body condition and the cause of death attributed during the necropsy, were collected and organized on Table 11.

Table 11. Gathered information from poxvirus positive animals, included in the phylogenetic tree. This information was collected in collaboration with the North Marine Mammal Stranding Network.

Nº	Species	Year	Necropsy date	Stranding site	Stranding type	Decomposition state	Age	Weight	Sex	Necropsy findings	Attributed cause of death
251	Delphinus delphis	/14	22/04/2014	Praia do Areão (Mira)	Dead	3 - Moderate decomposition	Juvenil >1ano	150	Female	Mutilated fluke; Right lung with a purulent lesion 10x5 cm; St3 with parasite cyst	Accidental capture
159	Delphinus delphis	/14	29/01/2014	Hospital, Praia do (Figueira da Foz)	Dead	3 - Moderate decomposition	Juvenil >1ano	160	Female	Adipose tissue dyed with blood throughout all its extension; Presence of abundant fluid in the abdominal cavity stained with blood; St3 with parasite cysts; Presence of food in St1	Accidental capture
160	Delphinus delphis	/14	30/01/2014	Esposende	Dead	3 - Moderate decomposition	Juvenil >1ano	185	Male	Several blood-stained organs	Probable accidental capture
162	Delphinus delphis	/14	30/01/2014	Esposende	Dead	3 - Moderate decomposition	Adult	200	Female	Broken beak; Dorsal musculature with rupture in the area posterior to the head; Trachea with foam; Parasite cysts in the lungs	Accidental capture
165	Delphinus delphis	/14	01/02/2014	São Pedro de Moel	Dead	3 - Moderate decomposition	Adult	239	Male	External exam: cord mark on the peduncle with laceration and blood; Healing ulcer in St1 3x1cm; Ruptured liver 5x5cm; Lungs with calcifications compatible with parasite cysts	Accidental capture
180	Delphinus delphis	/14	15/02/2014	São Pedro de Moel	Dead	3 - Moderate decomposition	Juvenil >1ano	200	Male	Beak fracture; Parasite cysts in abdominal cavity <10; St1 with ulcer scar; General organ congestion	Accidental capture
233	Tursiops truncatus	/12	12/04/2012	Praia de Mira	Dead	2 - Fresh		298	Male	Broken teeth; Hemorrhagic eyes; Blood-stained heterogeneous prescapular lymph node (LN); Adipose tissue in the dorsal area of the neck and jaw stained with blood; Trachea with foam and serous fluid; Hard and calcified cysts in the lungs and associated LN with heterogeneous consistency; Abdominal cavity with blood-tinged fluid; Piece of fishing net in the esophagus; St1 with large ulcer in regression; Kidneys presented an abnormal smell and dispersed gas bubbles; Bladder with hemorrhagic mucosa and urine with flocculant/cloudy material	Accidental capture
237	Delphinus delphis	/14	16/04/2014	Praia de Mira	Dead	2 - Fresh	Adult	201	Female	Left lung with areas of adhesion to the costal wall - presence of fibrin; Negative pressure right lung; 1 whole fish in the bronchus; Purulent associated lymph nodes, with increased consistency, enlarged and marbled appearence; Pericardium with adhesions to the left lung; St1 with a 5cm ulcer - presence of nematodes; Partially digested fish in the stomach;	Accidental capture
156	Delphinus delphis	/14	17/01/2014	Figueira da Foz	Dead	3 - Moderate decomposition	Adult	221	Male	Perfurating wound on the dorsal area; Alteration of 2 dorsal processes of lumbar vertebraes associated with a dermal lesion; Parasites in the lungs; 1 fish tail in a bronchus; Pericardium with a thickened and non transparent appearance; St1 and St2 with ulcers and subtancial parasitic load - nematodes; Digested fish in Stomach (St)1; Liver with parasite lesions and thickened walls	Accidental capture
262	Delphinus delphis	/14	07/05/2014	Gondarém, Porto	Dead	3 - Moderate decomposition	Juvenil >1ano	141	Female	Slightly increased prescapular LN; Crassicauda in the dorsal zone between muscle and tail; Foam in the trachea; St3 with parsitic cysts	Accidental capture
292	Delphinus delphis	/14	09/08/2014	Praia de Mira	Dead	3 - Moderate decomposition	Juvenil >1ano	163	Male	Aabdominal cavity with abundant blood-tinged fluid; Parasite cysts in the lungs	Accidental capture
295	Stenella coeruleoalba	/14	18/08/2014	Poço da Cruz, Mira	Dead	2 - Fresh	Juvenil >1ano	178	Female	Lungs with calcified lesions compatible with parasites; Hemorrhagic foci and very thin lung edges; Adrenal glands: enlarged marrow with cyst, hemorrhagic cortex; Abdominal cavity: medium load of parasite cysts; Esophagus+St2+duodenal ampulla with hemorrhagic mucosa; Blood-tinged pancreas	Sickness

Positive samples, included in the phylogenetic tree were gathered from 12 different individuals, collected within a 230 km range: the northern point being Esposende beach (Braga district) and the southern point being São Pedro de Moel beach (Leiria district) (Figure 18).

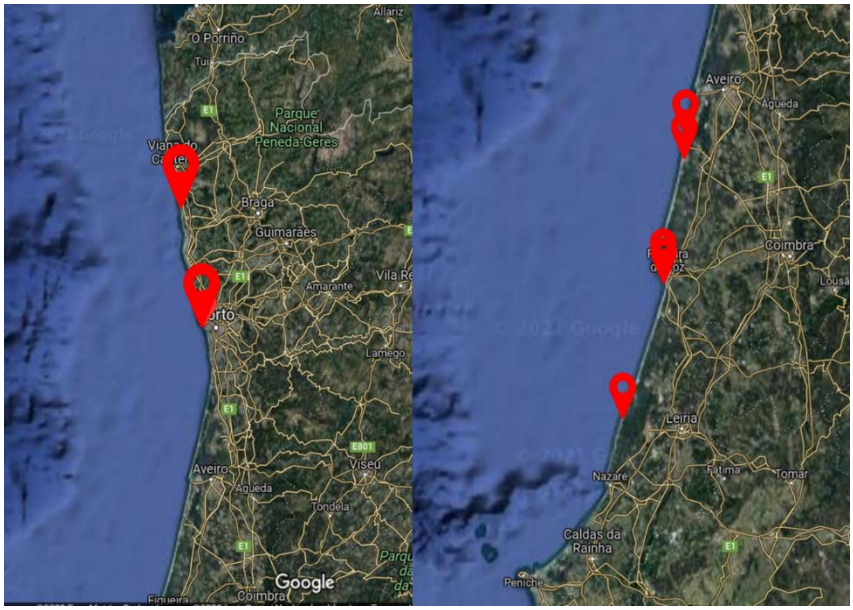


Figure 18. Geographic location of the strandings. Red pins mark the beaching site of the carcasses which originated the twelve samples with sequenceable viral DNA polymerase gene. Source: Google maps

All subjects were found stranded already dead, with a moderate (n=9, 75%) or fresh (n=3, 25%) body decomposition. The gross analysis shows a ratio between female and male subjects of 50/50. The percentage of juveniles was 58,3% (n=7), showing a slightly higher incidence than in adult animals. Necropsy findings were from a broad nature, however, lesions such as hematomas, fractures or parasitic forms, were consistent. These types of lesions, although expected on stranded animals, lead to the attribution of the cause of death to accidental capture in more than 90% (n=11) of the subjects. Only the animal SC295/14 presented pathological signs of generalized sickness.

4.2.2. Cetacean coronavirus survey

Concerning the coronavirus survey, primers and PCR conditions were primarly tested on three (n=3) coronavirus genera: alphacoronavirus, betacoronavirus and gammacoronavirus. Amplicons with expected molecular weight of 430 bp, were obtained in the three coronavirus genera tested (Figure 19), proving the system sensibility to all of the tested materials, which included vaccines and field isolates

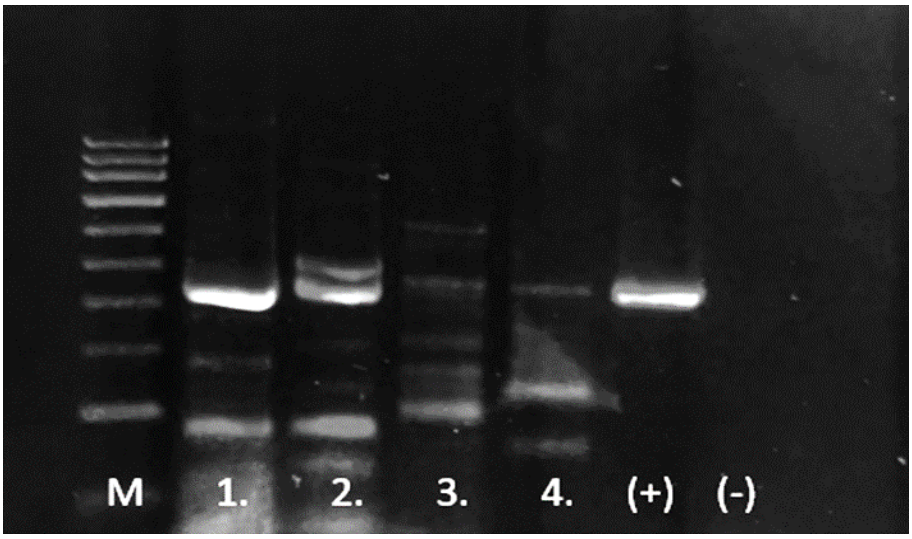


Figure 19. Coronavirus' system validation. (M) Molecular weight marker; (1) Gallivac IB88 vaccine; (2) Biovac IB vaccine (3) BovCoV vaccine (4) Goat field isolate positive for BovCoV; (+) Positive control; (-) Negative control. Original

The specificity and the wide coronavirus spectrum of the RT-nested PCR described by Holbrook et al, 2021, was confirmed after amplification of Canine enteric coronavirus (alphacoronavirus) Bovine coronavirus (betacoronavirus) and Infectious Bronchitis coronavirus (gammacoronavirus) IBV followed by direct sequencing (STABVIDA, Portugal). The nucleotide sequences are presented on Annexes (Table 14).

Bovine coronavirus isolate BOV-36/IND/2015 replicase protein gene, partial cds

Sequence ID: **MH753496.1** Length: 598 Number of Matches: 1

Range 1: 18 to 442

Score	Expect	Identities	Gaps	Strand	Frame
763 bits(413)	0.0()	421/425(99%)	0/425(0%)	Plus/Minus	

```
Query  4    CCATCATCACTAAAAATCATCATACTAAAATGCTTATTTAAAAATTCATAATATTCTGTG  63
            ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  442  CCATCATCACTCAAAATCATCATACTAAAATGCTTATTTAAAAATTCATAATATTCTGTG  383

Query  64   ACAAAGGTTGAATCAACCATATCACTTCTATACACATGTGAGTATAAGCGCTTCTGAAGA  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  382  ACAAAGGTTGAATCAACCATATCACTTCTATACACATGTGAGTATAAGCGCTTCTGAAGA  323

Query  124  GCACGTATACTCAAATCTTCAATCTTATTACCATTGCATGACATTAAAGCACATACATTG  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322  GCACGTATACTCAAATCTTCAATCTTATTACCATTGCATGACATTAAAGCACATACATTG  263

Query  184  GCTGAAACAGCTTGACATATGTTAAAAACTGAATTAGCAAAAGCAGTAGTTGCATCACCA  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  262  GCTGAAACAGCTTGACATATGTTAAAAACTGAATTAGCAAAAGCAGTAGTTGCATCACCA  203

Query  244  CTACTAGTGCCACCAGGCTTAACATAATAACAGCCACCACACATAACAATTTCACTCAAA  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  202  CTACTAGTGCCACCAGGCTTAACATAATAACAGCCACCACACATAACAATTTCACTCAAA  143

Query  304  ACTTGGGCGCATTCATTCGCAAGTCGATAAAACCTATCGCTTTGCGAACAACATGCCTCA  363
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  142  ACTTGTGCGCATTCATTCGCAAGTCGATAAAACCTATCGCTTTGCGAACAACATGCCTCA  83

Query  364  TGTTTTCGAGCCAAAACCAGACTACTAACAATACGTAGTATGTTTGGCATAGCGCGGTCA  423
            |||||||||||||||||||||||||||||||||||||||||||||||| || |||||||||
Sbjct  82   TGTTTTCGAGCCAAAACCAGACTACTAACAATACGTAGTATGTTTGGCATAGCACGATCA  23

Query  424  CATTT  428
            |||||
Sbjct  22   CATTT  18
```

Figure 20. Results from BLAST search from the sequence obtained through a goat field isolate. The sequenced is aligned with MH753496.1 with a 99% of identity, 100% query cover and a 0 E-value. Source: https://blast.ncbi.nlm.nih.gov/Blast.cgi.

The assignment of the nucleotide sequences after submission to BLAST (Basic Local Alignment Search Tool - NCBI), were in accordance with members of each coronavirus family (Figure 20).

Finally, a total of fifty-five (n=55) samples from five different species: *Delphinus delphis, Stenella coeruleoalba, Tursiops truncates*, *Phocoena phocoena* and *Kogia breviceps*, constituted by a pool of kidney and mesenteric lymph node in equal parts, were analysed through PCR assay (Figure 21).

No sample tested positive (0%) for Cetacean coronavirus (Graphic 5).

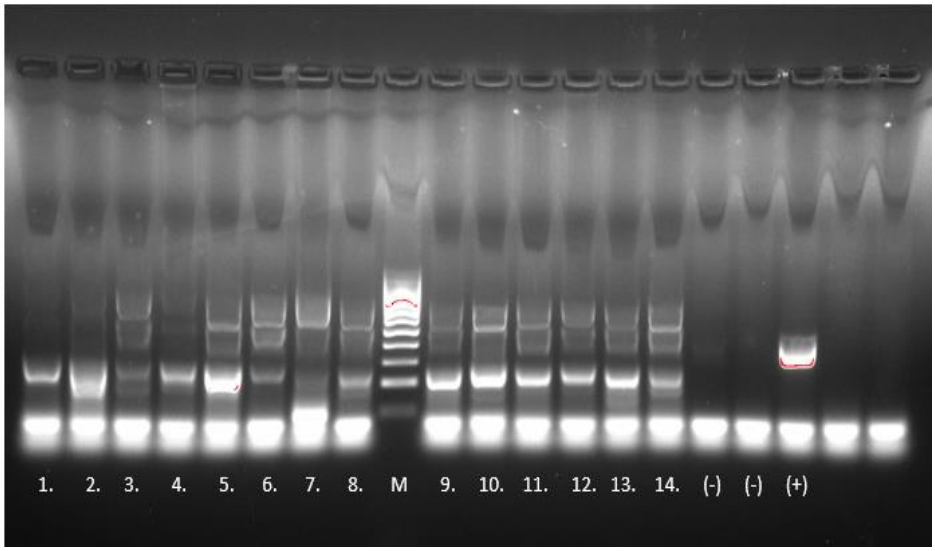
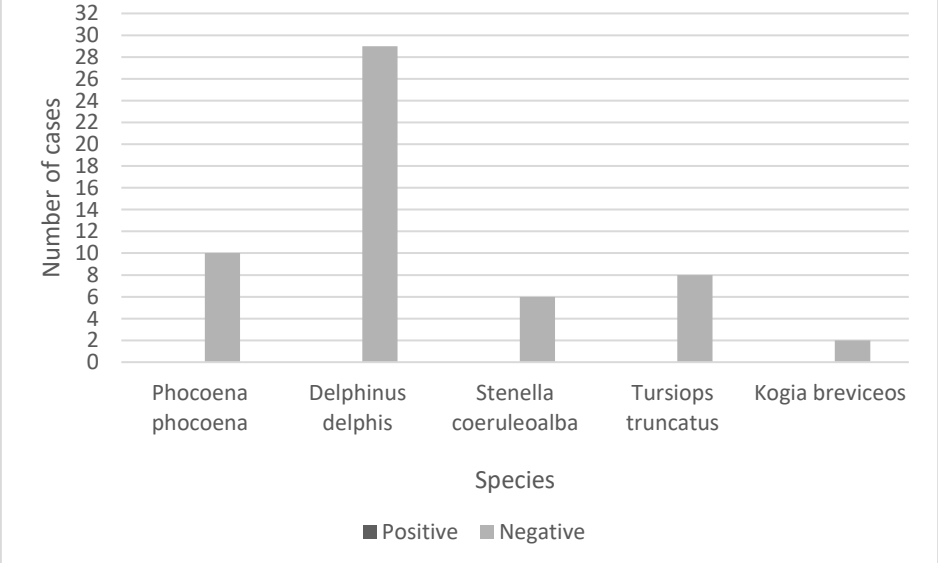


Figure 21. PCR amplification, using the system described by Holbrook et.al. (M) Molecular weight marker; (1-7; 11-14) *Delphinus delphis*; (8) *Tursiops truncates*; (9) *Stenella coeruleoalb;* (10) *Phocoena phocoena* samples; (+) Positive control; (-) Negative control. Original

Graphic 5. Results obtained through the Cetacean coronavirus survey using the PCR assay described by Holbrook et al. Distribution organized by species



5. Discussion

Concerning the Cetacean poxvirus survey, except for six samples, the majority of tissues were collected from animals stranded during the year of 2014.

Just a fraction of the animals (mammals or birds) that die at sea do actually strand (Bibby and Lloyd 1977; Peltier et al. 2012). The stranding rate can depend on several factors such as floatability, wind and climacteric conditions. Even when stranded, carcasses may not ever be found, may return to sea due to tidal variations or may also be reached by opportunistic scavengers prior to humans (Peltier et al. 2012). Since every coastline has particular characteristics, depending on space and time, establishing a stranding rate is highly conditioned.

The studies carried out during the LIFE+ MarPro project in Portugal, allowed to set a carcass detection rate, through several field experiences. It was estimated that, depending on the species, rates could vary from 2.18% (*Stenella coreuleoalba*) to 19.6% (*Balaenoptera acutorostrata*), with intermediate values of 11.9% (*Phocoena phocoena*) and 9.97% (*Tursiops truncates*) (Vingada and Eira 2018).

In 2014, an approximate number of 325 stranded cetaceans was registered. Since our survey identified 29/47 positive samples for Cetacean poxvirus, a percentage of 61,7%, it means that at least 8,9% of the populations present on Portuguese shores, on the year of 2014, was or had been infected with the virus.

Based on the above stated concerning stranding rates, we can expect the actual prevalence of the virus in the population to be substantially higher. Adding the fact that upon recovery by the North Marine Animal Stranding Network, many carcasses are categorized as highly decomposed (39,74%) and are not suitable for sampling, the underestimation is inherent.

On the other hand, it is important to highlight the fact that our baseline sample pool was biased. All of our materials belonged to a set of skin samples already compatible with Cetacean poxvirus infection.

The PCR assay used in the general survey, described by Sacristán et al. 2018, showed high sensibility (Figure 15), reducing the chances of having a false negative sample amongst the obtained results. Concerning specificity, since all the samples sent to sequence and blasted, came out as Cetacean poxvirus, having high query cover and identity percentage, the assay specificity was considered reliable. Specificity could not be fully assessed since, due to time and budget limitation, not all of the PCR products were sequenced.

The following set of tests, targeting the DNA polymerase, aimed the amplification of a genomic segment whose length allowed an acceptable relevant phylogenetic analysis. Coursing through the information gathered during the necropsy of the stranded

animals (Table 11), some information can be interpreted critically. Successful sequencing of the partial DNA polymerase gene (n=12) was obtained from individuals caught over a wide spatial range (230 km), covering the majority of the territory under North Marine Animal Stranding Network control. All animals were collected after stranding naturally, excluding DD237/14, whose death and beaching resulted from direct interaction with human activities, namely a traditional Portuguese fishing method – Xávega art. In all but individual SC295/14, alterations compatible with accidental capture were detected in the carcasses: blood-stained tissues, hemorrhages, hematomas, fractured facial bones, etc. Sample SC295/14 was collected from an animal which presented generalized sickness, with several organs showing signs of hemorrhage and inflammation. As expected, this makes the infection with Cetacean poxvirus not the attributed cause of death in 91% of the animals in question. Once again, it is notorious the impacts of human activity such as fishing and excessive harvesting on marine ecosystems. On current days, these activities are identified as the biggest threat to migratory marine species (Lascelles et al. 2014).

Upon necropsy, individuals (Table 11) presented a decomposition state between fresh (2) and moderate decomposition (3). The collection of samples from stranded carcasses, namely when directed to microbiology and virology, is of the maximum urgency, since *post-mortem* alterations can prevent viral identification/isolation (IJsseldijk, Brownlow, and S. Mazzariol 2019).

Distribution of sex and age was not significant. Virus identification could be performed in both male and female, in adults and in juvenile individuals. Juveniles are prone to show signs of infection, due to the loss of maternity immunity and lack of a long-lasting immunity to the virus (C.J. Hurst 2011). On the other hand, when adults show a high prevalence of infection, the health status of the population may be questioned. Even though the data collected by this study is not statistically relevant, further investigation should be interesting.

Analyzing the phylogenetic tree, the subgroup CePV-2 maintains an expected phylogenetic distance, corroborating the taxonomy established at the moment, which correlates CePV-2 with mysticetes and all the other subgroups with odontocetes (Bracht et al. 2006; Blacklaws et al. 2013; Fiorito et al. 2015; Sacristán et al. 2018). Further on, one sample clustered with CePV-6 (DD180/14) and the single sample from *Stenella coeruleoalba* (SC295/14), grouped within a branch joining CePV-1, 3, 5 and the unassigned CePV (KC409037). Two sequences (KC409037 and JN654445 - CePV-5) were obtained from *S. coeruleoalba* collected in the United Kingdom (UK), even though in different locations. The clustering with our sample SC295/14 reenforces the theory that dolphin species on the east North Atlantic ocean may form one single population

(Murphy et al. 2015) and explains the identification of genetically identical virus on northern and southern waters. However, the DQ071860 sequence (CePV-1) from a *S. coeruleoalba*, also clustered within this group, belongs to an individual from Florida, which interferes with the establishment of a pattern, since *S. coeruleoalba po*pulations from the western and eastern Atlantic Ocean do not share common grounds (Archer and Perrin 1999).

Concerning the remaining Portuguese samples (DD262/14, DD160/14, DD237/14, DD162/14, DD159/14, DD165/14, DD156/14, TT233/12, DD292/14, DD251/14), their grouping within a closed cluster, suggests the assignment of a 7th CePV group (CePV-7), following the characterization suggested by Barnett et al. 2015. Although having a bootstrap value of 59%, the genetic rift between this samples and the remaining sequences is clear.

Lastly, an apparent misleading evenness may be noticed at first sight. However, the presence of multiple cetacean species in shared clusters is persistent, raveling the hypothesis of species specific relations between cetacean species and Cetacean poxvirus. In general, and with the prementioned exceptions, the virus does tend to cluster according to geographical sites than within host species. The branching of sequences obtained from different animal species and geographical locations clearly hampers the establishment of an incidence pattern (Table 12).

Recently, the complete genome of CePV-1 was sequenced for the first time, through a sample obtained from an Indo-Pacific bottlenose dolphin (Tursiops aduncus) (Rodrigues et al. 2020), which will certainly help to understand the roll of certain encoded proteins on the virus' host range.

Concerning Cetacean coronavirus and our resultus, inumerous questions arise. An inter-species jump between birds and cetaceans may have occurred. Birds are natural pools that supply viral genes during the development of new species and viruses for interspecies transmission (Rahman et al. 2021). Within the gammacoronavirus genera, one of the most representative virus is avian coronavirus, which covers the highly contagious infectious bronchitis viruses (IBV) in chickens, and similar viruses on domestic birds. IBVs have also been identified in healthy wild birds, showing their capacity to serve as reservoirs between domestic and free-living birds (Miłek and Blicharz-Domańska 2018).

Wild aquatic birds belonging to different orders such as *Anseriformes*, *Charadriiformes* (including gulls) and *Pelacaniformes*, have been identified as positive for gammacoronvairuses and already confirmed by different authors (Muradrasoli et al. 2010; Wille et al. 2018; Canuti et al. 2019).

The association between seabirds and cetacean species is known since the 19th century. One of the main reasons causing this phenomenon is that, as predators, they share food supplies. Some advantages came along with this evolution: easier long-distance location of prey, concentration of food prey, increased accessibility of prey by bringing to surface and by panicking/stunning shoal, availability of dead prey either whole or parts on surface (Evans 1982). However, the coexistence of these animals forms a probable and viable crossing point for virus and other pathogens.

In a 2019 study, a new gull CoV was actually discovered. After a phylogenetic analysis, the resulting phylogenetic tree placed this new virus as one of the closest to the described Cetacean coronavirus genetically wise (Figure 22) (Canuti et al. 2019).

The Cetacean coronaviruses previously reported were identified from individuals in a condition of captive (Mihindukulasuriya et al. 2008; Patrick C. Y. Woo et al. 2014) or semi captive (Wang et al. 2020). The regime of semi captive is used by this study when referring to animals belonging to the US Navy Marine Mammal Program. Even though the animals in this program are kept in destined facilities, they are frequently involved in exercises on open waters, having direct contact with natural habitats.

The origin of the infection is until today, unknown. How the virus spread from enclosured bottlenose dolphins in Hong Kong to semi-captive bottlenose dolphins in USA is unclear, but taking in consideration the genetic similarity between the 2 strains (HK-BdCoV and US BdCoV) (Wang et al. 2020), the existing possibility of the virus circulating in wild environments is considerably high.

The active role of birds in the virus circulation is also a possibility. Due to their migratory nature and ubiquity, wild birds are known to be exceptional virus dispersers. We cannot also forget the massive amplification capacity that birds or poultry represent, since they comprise three times the biomass of wild birds on the planet (Wille and Holmes 2020).

Returning to our negative survey results, another defining point was the selection of the materials to test. Cetacean coronavirus was identified in asymptomatic individuals (Patrick C. Y. Woo et al. 2014), in lethargic individuals with diarrhea (Wang et al. 2020) and in a lethal case, where the postmortem examination revealed generalized pulmonary disease and acute liver failure (Mihindukulasuriya et al. 2008). This diversity of clinical signs difficulted the samples' choice.

Due to the impossibility of direct feces collection, the decision of analyzing the mesenteric lymph nodes took in consideration the essential role that they represent in intestinal immunity (Pabst et al. 2009); and kidney was also considered due to what is performed when dealing with feline coronavirus (Sharif et al. 2010).

The present survey was performed on tissues belonging to strandings occurring between 2012 and 2021, increasing the possibility to encounter a possible outbreak of the virus in the wild. The PCR system used and described by Holbrook et al. 2021, proved to be sensitive, identifying members of 3 out of 4 coronaviruses' genera (alpha-, beta- and gammacoronavirus), from field samples or vaccines. After BLAST, the specificity was also confirmed (Figure 20). Taking in consideration the sensitivity tests performed by the original author Holbrook et al. 2021 where, according to the type of CoV, sensitivities would vary between 1–100 copies/µL, the system should be capable of identifying Cetacean coronavirus, unless the virus titers were considerable low.

Although we did not successfully identify the virus in Portuguese waters, further studies would be of considerable importance. The attempt here described of identifying Cetacean coronavirus in free range, stranded, dead cetaceans, was in some way innovative. In future studies, the sampling method is a variant that should be considered. Testing materials such as lung and liver could be an interesting update.

Viruses are ubiquitous and the human interaction with animals has been appointed as a primary risk factor for several high impact zoonoses and spillovers (Li et al. 2019). Although the general concept of One Health is widely accepted, multi-sectoral cooperation in the surveillance and control of emerging infectious diseases is still a challenge, and will continue to be as long as the gap between the fields of animal and human health exist (Ryu et al. 2017).

6. Final considerations

Presently, the One Health approach is a in vogue concept. The global changes that the last decades brought and their complex patterns, evidenced the inextricable interconnection of humans, pet animals, livestock and wildlife and their social and ecological environment. An integrated approach to human and animal health and their respective environmental context is considered the go to method for a harmonious future (Zinsstag et al. 2011).

Understanding that human and animal health are a single entity, promotes research. Surveys should be frequent, allowing the increase of the knowledge on complex biotic relationships between distinct pathogens and their wild animal reservoirs (Cupertino et al. 2020). More studies should be performed on pathogens circulating among wild animals. Comparative analysis between wildlife and domestic or human pathogen strains is, in many cases underestimated and, consequently, epidemiological cycles of infectious diseases are not well assessed in populations of concern worldwide (Ryser-Degiorgis 2013).

The work developed by Stranding Networks around the world is of major importance. In Portugal, the tasks performed by the several regional networks allows the expansion of knowledge on biological, ecological and behavioural matters. The foundation of a tissue bank from stranded animals is crucial to projects like this one. Monitoring wildlife populations, whether retrospectively or in real time, is a tool that should be taken advantage of.

Cetacean poxvirus was successfully identified and genetically characterized in this study. As far as the author knows, it was the first time that molecular analysis was used for the virus identification in Portugal. Although, due to time and resources constrains, the study does not represent statically relevant results, it was able to bring some insights about the virus distribution within wild populations.

Looking up at future projects, testing a wider pool of samples for real prevalence calculations should be a point to take in consideration. Also, the present study could not establish a correlation between the macroscopic aspects of the skin lesions and possible genetic variations within different Cetacean poxvirus. There are numerous questions still unanswered about this virus, however advances about the virus genome (Rodrigues et al. 2020) could open new horizons.

Concerning the Cetacean coronavirus survey, there is an obvious need for future investigations. Coronavirus are a family of virus with undeniable zoonotic behaviour, being the protagonist of the spill over event, which resulted in the present pandemic.

Understanding the virus behaviour in the wild, whether in beta-, alpha-, delta- or gammacoronavirus genera, may represent an open door for future preventive measures. The lack of positive results in the present survey, suggests a change of protocol. In the future, the biologic tissues put to test could be a focal point in order to successfully identify the agent in wild environments.

Viruses are ubiquitous, and the seas represent no exception. Further molecular research in both viruses will be required to fully understand the extension of their distribution and pathogenic impact.

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8. Annexes

Table 52. Virus presented in the phylogenetic tree designed by Barnett et al. Organized by GenBank accession number, species, collection location and year.

Virus	Ac. Code	Specie	Local	Year	
CePV-6	JN654440	Delphinus delphis	United Kingdom	2010	Barnett
CePV-6	JN654441	Delphinus delphis	United Kingdom	2009	Barnett
CePV-6	JN654443	Delphinus delphis	United Kingdom	2011	Barnett
CePV-6	KC242456	Delphinus delphis	United Kingdom	2012	Barnett
CePV-6	JN654442	Delphinus delphis	United Kingdom	2009	Barnett
CePV-5	AY952950	Tursiops truncatus	USA	2005	Bracht
CePv-5	JN654445	Stenella coeruleoalba	United Kingdom	2008	Barnett
CePv-5	KC409049	Pocoena phocoena	United Kingdom: Humberside	2001	Blacklaws
CePv-4	KC242459	Pocoena phocoena	United Kingdom	2012	Barnett
CePv-4	KC242457	Pocoena phocoena	United Kingdom	2012	Barnett
CePv-4	JN654444	Pocoena phocoena	United Kingdom	2009	Barnett
CePv-4	KC242458	Pocoena phocoena	United Kingdom	2011	Barnett
CePv-4	KC409036	Pocoena phocoena	United Kingdom: Northumberland	2007	Barnett
CePv-3	AY463006	Tursiops aduncus	Hong Kong	2003?	Bracht
CePv-1	DQ071860	Stenella coeruleoalba	USA: Florida	2005?	Bracht
CePv-1	AY463007	Tursiops aduncus	Hong Kong	2003?	Bracht
CePv-1	DQ071862	Steno bradensis	USA: Florida	2005?	Bracht
CePv-1	DQ071858	Tursiops truncatus	USA: Florida	2005?	Bracht
CePv-1	DQ071856	Tursiops aduncus	Hong Kong	2005?	Bracht
CePv-1	AY463004	Steno bradensis	USA: Florida	2003?	Bracht
CePv-2	AY846759	Balaena mysticetus		2004?	Bracht
	KU726612	Tursiops truncatus	Brazil	2014	Sacristan
	KM000064	Eubalaena australis	Argentina	2013	Fiorito
	KC409037	Stenella coeruleoalba	United Kingdom: Dorset	2000	Blacklaws

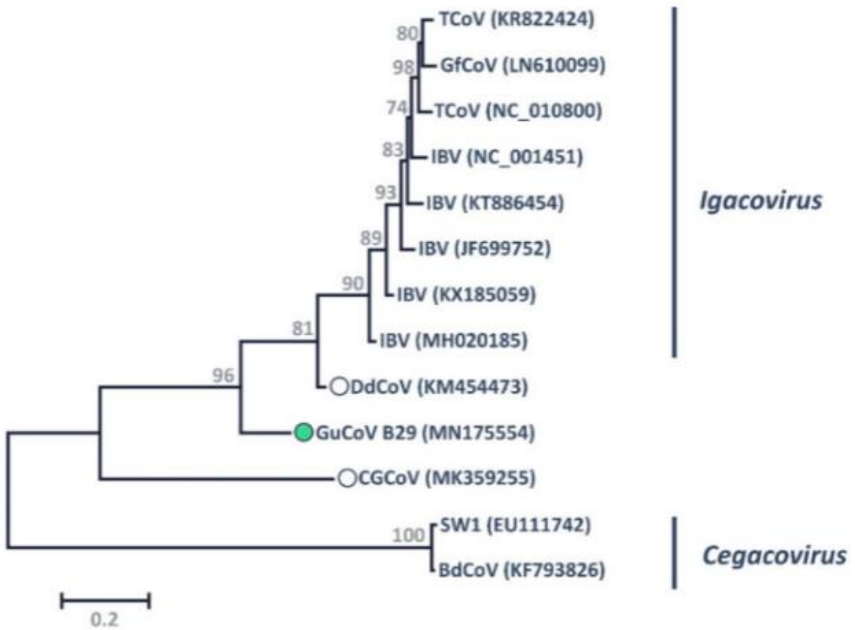

Figure 22. GuCoV B29 phylogenetic tree. The phylogenetic placement of the Gull coronavirus (GuCoV B29) within the genus Gammacoronavirus, with relative proximity to cetacean coronavirus. Source: Canuti et al. 2019

Table 13. Sequences obtained through Sanger sequencing during the Cetacean poxvirus survey. Organized by sample identification, sequenced gene and respective nucleotide sequence

SAMPLE	SEQUENCE	GENE
TT233/12	CCGTAAACAGAATTTGCAACTATTTTATAAGTATACTGTATAGAGTCATATATTGCTTTTTCTGTGCAATCATTAGTGGATTTTAATAATCTTTTGTATTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACATTAGGATACAAACTATTGTAGTCAAAGATTAAAACATTATTACTAAACATCTTTTGTTTCGGCATAAATACTTTACCTCCCTCGTAAGGAAGTTTTTGTTGTTTATATTCAGATCTATATAGGATGATTTTTGTTTCTAGTAACAACTTAAGTAAAGGAC	DNApol
SC295/14	AATAATCTTTYGTATTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAGATTCTTAAAACGTGGCTCACAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATACATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGGTTACCGAATAGGCACACATTAGGATACAAACTATTATAGTCAAAGATTAAAACATTATTACTAAACATTTTTTGTTTCGGCATAAATACTTTAACCTCCCT	DNApol
DD251/14	TTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACAACAGATCTAAGTGATAAAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACATTAGGATACAAACTATTGTAGTCAAAGATTAAAACATTATTACTAAACATCTTTTGTTTCGGCATAAATACTTTACCTCCCT	DNApol
DD159/14	TTTTAATAATCTTTTGTATTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACATTAGGATACAAACTATTGGAGTCAAAGATTAAAACATTATTACTAAACATCTTTTGTTTCGGCATAAA	DNApol
DD160/14	TTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGC	DNApol
DD162/14	TATTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACATTAGGATACAAACTATTGTAGTCAAAGATTAAAACATTATTACTAAACATCTTTTGTTGCGGCATAAATACTTTACCT	DNApol
DD165/14	TTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACATTAGGATACAAACTATTGTAGTCAAAGATTAAAACATTATTACTAAACATCTTTTGTTTCGGCATAAATACTTTACCTCCCT	DNApol
DD180/14	AGGTATAGTGCCTCAACTTCTCTATCGAATATGGTATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCTAAT	DNApol
DD237/14	TATTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACATTAGGATACAAA	DNApol
DD156/14	AATAATCTTTTGTATTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACATTAGGATACAAACTATTGTAGTCAAAGATTAAAACATTATTACTAAACATCTTTTGTTTCGGCATAAATACTTTACCTCCCTCGTAAGGAAGTTTTTGTTGTTTATATTCAGATCTATATAGGATGATTTTTGTTTCTAGTAACAACTTAAGTAAAGGACCTTTTATTAACGTACTAGCT	DNApol
DD262/14	ATCTTTTGTATTTTGCACGTTCTGTTAAGAATCGTCTTAGTATTCTAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATGTTTCTGGTGACAGATTGCCGAATAGGCACACAT	DNApol
DD292/14	TAGGTATAGTGCCTTCAACTTCTCTATCGAATATGGATATTTCAGAAATTAAATTCTTAAAACGTGGCTCGCAATGTACAACTATATATCTAGGTGATGGAAATTTCTTTTTTATTTCCTGAATATTAATCTCAAGTTCTAATATATTATTACTAACAACTACACCTACTAATG	DNApol

Table 14. Sequences obtained through Sanger sequencing during the Cetacean coronavirus survey. Organized by genus, virus and respective sequence.

GENUS	VIRUS	SEQUENCE
Gammacoronavirus	Infectious bronchitis (IBV)	CAGTGTGATGGATATCTGCAGAATTCGGCTTGACTACCCAAAATGTGATAGAGCAATGCCCAATTTGT TGCGTATTGCAGCATCTTTGGTACTTGCTCGTAAACACACTAATTGTTGTACTTGGCCTGAACGCATTT ATAGGTTGTATAATGAATGCGCGCAGGTTTTATCTGAAACTGTCTTAGCTACAGGTGGTATTTATGTG AAACCTGGTGGCACTAGCAGTGGTGATGCTACTACTGCTTATGCAAACAGTGTTTTTAATATAATACA AGCCACATCTGCTAATGTTGCGCGTCTTTTGAGTGTTATAACGCGTGATATTGTCTATGATGACATTAA AGACCTGCAGTACGAATTATACCAGCAGGTTTATAGGCGAGTCAACTTCGACTCGGCCTTTGTTGAAA AGTTCTATTCTTACTTATGTAAGAATTTTTCACTCATGAT
Betacoronavirus	Bovine coronavirus	CTTCCATCATCACTAAAAATCATCATACTAAAATGCTTATTTAAAAATTCATAATATTCTGTGACAAAG GTTGAATCAACCATATCACTTCTATACACATGTGAGTATAAGCGCTTCTGAAGAGCACGTATACTCAA ATCTTCAATCTTATTACCATTGCATGACATTAAAGCACATACATTGGCTGAAACAGCTTGACATATGTT AAAAACTGAATTAGCAAAAGCAGTAGTTGCATCACCACTACTAGTGCCACCAGGCTTAACATAATAAC AGCCACCACACATAACAATTTCACTCAAAACTTGGGCGCATTCATTCGCAAGTCGATAAAACCTATCG CTTTGCGAACAACATGCCTCATGTTTTCGAGCCAAAACCAGACTACTAACAATACGTAGTATGTTTGG
Alphacoronavirus	Canine Coronavirus	CATCAGATAGAATCATCATAGAAAAGTGTTTTCTTAGATAGCTGAAATATTCAACAACAAATTCTTCGT CAATACTGCTACTACGATAGCAATTGTCGTAGATTTTACGTTGTATGGATTTCACTGTAACATTGTTAC AAGCGTTTGAATCAACTCCCAAAAGCTTATTAACATTAGCAGAAACAGCTTGAAAGATGTTAAAAGCT GAGTTAGCATAAGCTGTAGTACCATCACCACTAGTTGTACCACCAGGTTTTATGTAAAAACCACCTGT GCAATGAACAACTTCTGTGAGTACTTGAGCTAACTCATTAGAGAGTCGATAGAATCTATCACTATGTG TACAACAACCAACGTGCTTAGAACCCAGTATCATGGCAGAAGCCATTCTAATCATATTGGGTAAAGCA CGATCGCACTTAGGATAATCCC

Table 65. List of materials tested during the Cetacean poxvirus survey

Nº	Species	Year		Sacristã	To sequence	DNApol	To sequence
251	DD	/2014	x	pos		pos	x
155	DD	/2014	x	pos			
159	DD	/2014	x	pos		pos	x
160	DD	/2014	x	pos		pos	x
161	DD	/2014	x	pos	x		
162	DD	/2014	x	pos		pos	x
165	DD	/2014	x	pos		pos	x
166	DD	/2014	x	neg			
175	DD	/2014	x	pos			
180	DD	/2014	x	pos		pos	x
191	DD	/2013	x	neg			
200	SC	/2013	x	neg			
204	DD	/2014	x	pos	x		
219	DD	/2014	x	neg			
225	DD	/2014	x	neg			
233	TT	/2013	X	pos	x	pos	x
236	DD	/2014	x	neg			
237	DD	/2014	x	pos		pos	x
238	DD	/2014	x	neg			
239	DD	/2014	x	neg			
240	DD	/2014	x	pos		pos	x
241	DD	/2014	x	neg			
242	DD	/2014	x	pos		neg	
247	DD	/2013	x	neg			
248	DD	/2014	x	neg			
249	DD	/2014	x	pos		pos	
255	DD	/2014	x	pos			
156	DD	/2014	x	pos		pos	x
262	DD	/2014	x	pos		pos	x
263	DD	/2014	x	pos		pos	x
264	DD	/2014	x	neg			
265	DD	/2014	x	neg			
266	DD	/2014	x	neg			
270	PP	/2014	x	pos		neg	
271	PP	/2014	x	pos		neg	
272	DD	/2014	x	neg			
279	DD	/2014	x	pos		pos	
280	SC	/2014	x	pos		pos	
285	PP	/2014	x	neg			
289	DD	/2014	x	pos		pos	x
292	DD	/2014	x	pos		pos	x
293	DD	/2014	x	pos		neg	
295	SC	/2014	x	pos		pos	x
296	PP	/2014	x	pos		pos	
298	SC	/2011	x	neg			
308	DD	/2014	x	pos	x		
250315	SC	/2015 (?)	x	neg			

Table 76. List of materials tested during the Cetacean coronavirus survey

Nº	Species	Necropsy date	LNmesent	[ ] ng/µl	10µl	cDNA/µl	Kidney	[ ] ng/µl	10µl	cDNA/µl
300	TT	/2015	X - Extraído				X - Extraído	26,7	267	13,35
164	DD	/2012					X - Extraído	29	290	14,5
263	TT	/2012	X - Extraído				X - Extraído	30,8	308	15,4
197	DD	/2021					X - Extraído	39,1	391	19,55
177	DD	/2021					X - Extraído	42,7	427	21,35
231	DD	/2015	X - Extraído	36,6	366	18,3	X - Extraído	51,3	513	25,65
27	TT	/2014					X - Extraído	59	590	29,5
227	PP	/2014	X - Extraído	97,6	976	48,8	X - Extraído	61,7	617	30,85
185	PP	/2013	X - Extraído	369,8	3698	184,9	X - Extraído	62,9	629	31,45
233	TT	/2012	X - Extraído				X - Extraído	65,6	656	32,8
211	DD	/2013	X - Extraído	200	2000	100	X - Extraído	71,9	719	35,95
266	KB	/2014					X - Extraído	73	730	36,5
144	DD	/2015	X - Extraído	129,5	1295	64,75	X - Extraído	79,6	796	39,8
163	DD	/2016					X - Extraído	84	840	42
9	SC	/2014					X - Extraído	90,5	905	45,25
192	DD	/2021					X - Extraído	102,8	1028	51,4
179	DD	/2014					X - Extraído	103,7	1037	51,85
200	DD	/2014					X - Extraído	108	1080	54
214	PP	/2015	X - Extraído	90,4	904	45,2	X - Extraído	115,6	1156	57,8
296	PP	/2014					X - Extraído	124,8	1248	62,4
261	DD	/2013	X - Extraído	69,8	698	34,9	X - Extraído	130,6	1306	65,3
271	KB	/2012					X - Extraído	131	1310	65,5
227	PP	/2015					X - Extraído	132	1320	66
163	DD	/2012					X - Extraído	132,7	1327	66,35
211	DD	/2014					X - Extraído	136	1360	68
178	DD	/2016	X - Extraído	398,3	3983	199,15	X - Extraído	137,7	1377	68,85
211	DD	/2021					X - Extraído	137,9	1379	68,95
212	DD	/2021					X - Extraído	140,4	1404	70,2
292	DD	/2014	X - Extraído	154,1	1541	77,05	X - Extraído	142,9	1429	71,45
198	TT	/2021					X - Extraído	144,6	1446	72,3
222	TT	/2013	X - Extraído				X - Extraído	149,7	1497	74,85
155	DD	/2016					X - Extraído	150	1500	75
71	TT	/2014					X - Extraído	151,3	1513	75,65
206	SC	/2021					X - Extraído	152,1	1521	76,05
194	DD	/2021					X - Extraído	155,5	1555	77,75
193	DD	/2021					X - Extraído	159,5	1595	79,75
196	DD	/2021					X - Extraído	162,8	1628	81,4
156	DD	/2016	X - Extraído	199,3	1993	99,65	X - Extraído	163,4	1634	81,7
213	DD	/2021					X - Extraído	165,8	1658	82,9
216	PP	/2015	X - Extraído	203,6	2036	101,8	X - Extraído	168,8	1688	84,4
143	DD	/2015					X - Extraído	170	1700	85
152	PP	/2015					X - Extraído	170	1700	85
365	SC	/2014					X - Extraído	170,6	1706	85,3
213	DD	/2014	X - Extraído	407,4	4074	203,7	X - Extraído	178,3	1783	89,15
269	TT	/2012	X - Extraído				X - Extraído	181,8	1818	90,9
277	PP	/2014					X - Extraído	202	2020	101
140	DD	/2012					X - Extraído	212	2120	106
199	DD	/2012					X - Extraído	213	2130	106,5
280	SC	/2014					X - Extraído	237	2370	118,5
254	PP	/2014	X - Extraído	194,2	1942	97,1	X - Extraído	239,7	2397	119,85
169	DD	/2021					X - Extraído	248,5	2485	124,25
180	SC	/2016					X - Extraído	267	2670	133,5
173	PP	/2021					X - Extraído	282,6	2826	141,3
200	SC	/2013					X - Extraído	330	3300	165
156	DD	/2021					X - Extraído	343,9	3439	171,95
ROTTA								48	480	24
BIROS								115,9	1159	57,95
Gallivac88								209.4	2094	104,7
Bio 120								80	800	40
Bov (3498/07)								100,9	1009	50,45
Cap (1178/07)								7,3	73	3,65