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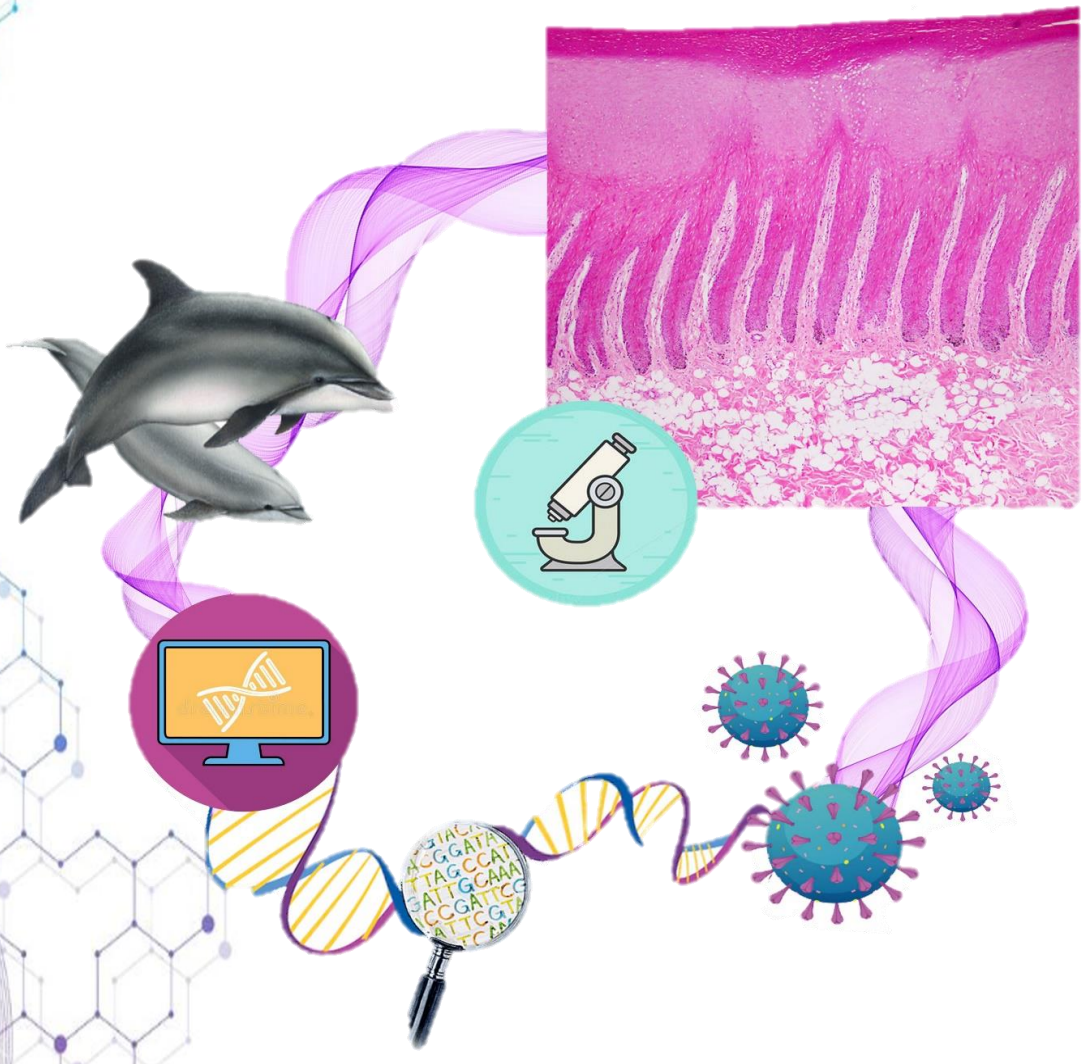
TESIS DOCTORAL

CETACEAN POXVIRUS SKIN DISEASE: A POTENTIAL BIOINDICATOR OF HEALTH AND WELFARE IN MARINE MAMMALS



SIMONE ANDREA SEGURA GÖTHLIN

DOCTORADO EN SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA

LAS PALMAS DE GRAN CANARIA

NOVIEMBRE 2023

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ESCUELA DE DOCTORADO – UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

LAS PALMAS DE GRAN CANARIA

SEPTIEMBRE 2023

Tesis presentada por: Dª Simone Andrea Segura Göthlin.
Dirigida por: Dra. Eva Sierra Pulpillo y por el Dr. Manuel Arbelo Hernández.

La Directora:	El Director:	La Doctoranda:
(Firma)	(Firma)	(Firma)

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

D. ANTONIO FERNÁNDEZ RODRIGUEZ, COORDINADOR DEL PROGRAMA DE DOCTORADO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha / / , tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "Cetacean poxvirus skin disease: a potential bioindicator of health and welfare in marine mammals" presentada por la doctoranda Dª Simone Andrea Segura Göthlin y dirigida por la Doctora Eva María Sierra Pulpillo y el Doctor Manuel Antonio Arbelo Hernández.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a de de dos mil veintitrés.

To the most precious thing that I have in life, to my guides, to my shelter; my parents.
Without you this would have been meaningless.

To those who know, understand, and support me the best; my sisters.
Success cannot be without you, but failure is guaranteed.

To my grandmother, who always have cared for me, no matter the distance between us.
To my grandfather, who has taught me to keep smiling and that any time is perfect to laugh, no matter how difficult the journey may sometimes be.

To my best friend, to my companion in this journey called life, to my partner in crime, to the love of my life, Besay.

And last but not least, to my other half, to my eyes, my footprints, and my shadow; Guayra.
The one who has taught me to love unconditionally.

Don’t rush the process,

you’ll get there at the right time.

As the sunset’s red when merging into the blushing sea.

As the wild waves reaching the shore to find the stillness of the wind.

As the flowers know that part of blossoming comes with rest.

As the light of a candle, before wasting away among the shades,

it has guided you to where you are.

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1.

INTRODUCTION AND OBJECTIVES

To date, cetaceans' skin has been considered a promising tool for gaining a better comprehension about a wide variety of features related to these marine mammals, becoming, therefore, the purpose of research for a broad range of scientific fields (Schick et al., 2013; Barratclough et al., 2019a). Among the wealth information that it can provide, cetaceans' skin is a key element for the evaluation of both animals' and ecosystems health status (Wilson et al., 1999; Van Bressem et al., 2009; Mouton and Botha, 2012; Barlow et al., 2019). Correspondingly, long-term surveys of their skin condition enable identifying potential anthropogenic threats in the wild such as for toxic contaminants (Aubail et al., 2013; Mancia et al., 2018; Zanuttini et al., 2019; Baini et al., 2020), marine traffic effects or fishery activities interactions, as well as foreign body entanglements due to marine pollution, between other (Van Bressem et al., 2007; Butterworth, 2017; Leone et al., 2019; Fossi et al., 2020; Puig-Lozano et al., 2020; Womersley et al., 2021). The continued exposure to all the above-mentioned distressful situations have arguably been suggested to cause immunosuppression and increased infectious disease susceptibility (Van Bressem et al., 2009; Bossart, 2011; Miller et al., 2018; Koch et al., 2018; Barratclough et al., 2019b; Bossart et al., 2019).

Accordingly, skin diseases play an essential role in gaining an approximate understanding of the general status of those marine mammals, being their study in relation to cetaceans' health one of the most documented approaches so far (Maldini et al., 2010; Mouton and Botha, 2012; Romero and Keith, 2012; Gonzalvo et al., 2014; Nelson et al., 2015). A considerable diversity of microorganisms with opportunistic pathogenicity capacities affects cetaceans' skin, generally taking advantage of prior damaged skin to penetrate and cause infection (Mouton and Botha, 2012; Bartlett et al., 2016). For instance, secondary bacterial and fungal skin infections are often a complication in cetaceans' cutaneous wounds, especially in those that are ulcerated, worsening, and sometimes, considerably hindering the skin healing process. Additionally, the latter combined with human impacts causes negative consequences in the health of those marine mammals, considerably affecting animals' biological functions (Higgins, 2000; Melero et al., 2011; Esperón et al., 2012; Ueda et al., 2013; Bossart et al., 2019; Duignan et al., 2020). Above all, for these aquatic mammals, several studies have hereto reported an increase of the frequency of viral skin diseases, whose development over time provides an insight of host health and immunologic responses both on an individual and overall population basis (Barr et al., 1989; Smolarek et al., 2006; Barnett et al., 2015; Fiorito et al., 2015; Dagleish et al., 2021).

Among viral skin infections, Tattoo Skin Disease (TSD) has significantly been reported in many cetacean species due to be visually distinguished from other skin disorders (Harzen and Brunnick, 1997; Van Bressem et al., 2003; Fury and Reif, 2012; Hart et al., 2012). This is due to cutaneous manifestations of reported poxvirus infections in cetaceans show a characteristic appearance widely known as “tattoos”, corresponding to lesions with flat or slightly depressed centre with a stippled pattern in the interior and delimited black margins (Figure 1) (Geraci et al., 1979; Bossart and Duignan, 2018; Powell et al., 2018a). Depending on their stage of evolution, these skin lesions may darken or appear completely pale, losing their margins. Despite the fact that to date this viral disease has not been proven to substantially have the pathogenic capacity to cause cetaceans’ deaths, it has been proposed that its emergence is related to individuals with affected health and/or with impaired immune system as a consequence of their disability to correctly cope with a constant unbalanced marine environment (Van Bressem et al., 2003; Fury and Reif, 2012; Cocumelli et al., 2018; Koch et al., 2018). In accordance with the latter, it is distinctive that tattoo lesions evolve and persist on cetaceans’ skin in an independently and indefinitely manner as well as recurrently disappear to later reappear (Geraci et al., 1979; Van Bressem et al., 1999; Mouton and Botha, 2012; Fiorito et al., 2016; Powell et al., 2020). Due to all the above mentioned, poxvirus in cetaceans may be considered a potentially useful general health indicator in those marine mammals (Van Bressem et al., 2009; Van Bressem et al., 2015).

Figure 1.Tattoo skin lesions in different stages of evolution located on the melon and tip from a stranded bottlenose dolphin (*Tursiops truncatus*) in Tenerife, Canary Islands. Image provided from the Animal Health and Food Safety Institute (IUSA), University of Las Palmas de Gran Canaria (ULPGC), Canary Islands, Spain.

Despite being broadly described, there is limited genetic information to correctly ascribe poxvirus infecting cetaceans. Previous reports suggest that they belong to an unassigned genus of the subfamily *Chordopoxvirinae,* being tentatively classified in a new genus known as cetacean poxvirus (CePV), that include at least two subgroups: cetacean poxvirus 1 (CePV-1) infecting odontocetes, and cetacean poxvirus 2 (CePV-2), infecting mysticetes (Bracht et al., 2006; Blacklaws et al., 2013; Rodrigues et al., 2020). The scarce sequencing of this viral agent is mostly due to the considerable ease to distinguish its characteristic cutaneous manifestation, leading to the inconsistent attempt to relate the presence of the pathogen in these lesions without performing diagnostic methods to corroborate infection (Bearzi et al., 2009; Van Bressem et al., 2017; Powell et al., 2018b; Stylos, 2019). Consequently, apart from the fact that this may lead to inherent assumptions with lack of scientific basis, it limits the knowledge of whether cetacean poxvirus may be present in skin manifestations other than tattoo lesions or whether concomitant pathogens may be associated with these lesions (Melero et al., 2014). Only few studies have developed sensitive methods to successfully detect poxvirus in cetaceans, such as different PCR techniques (for DNA polymerase and DNA topoisomerase I genes) which is up to now considered the reference diagnostic method for CePV-1 detection (Blacklaws et al., 2013; Cocumelli et al., 2018; Sacristán et al., 2018; Sacristán et al., 2018; Luciani et al., 2022). Complementarily, describing acidophilic intracytoplasmic inclusion bodies in epidermal cells through histology as well as identifying viral particles by transmission electron microscopy are often performed in order to serve as supportive procedures that enhance and strengthen the diagnosis (Sacristán et al., 2018; Luciani et al., 2022). In either way, skin samples from cutaneous lesions suspected of being infected with CePV are required to perform the above-mentioned techniques.

Generally, skin biopsies are the method of choice to collect skin samples from cutaneous lesions in cetaceans (Parsons and Durban, 2003; Gales et al., 2009; Romero and Keith, 2012; Noren and Mocklin, 2012). In the wild, remote biopsy techniques have been developed and used in multiple studies over recent decades, usually handled either with crossbows or modified riffles, both with stainless steel sampling tips and darts (Krutzen et al., 2002; Sinclair et al., 2015). However, the accessibility to free-ranging cetaceans in the marine environment is logistically complex, time-consuming, and relative expensive, becoming the skin sampling very difficult to carry out in many situations (Gales et al., 2009; Romero and Keith, 2012; de Mello and de Oliveira, 2016; Boggs et al., 2019). In addition to this last, there is no clear evidence about the possible negative impact that these biopsy

sampling methods may suppose in wild cetaceans, having been reported, for now, aberrant behaviours during the pursuit and the sampling, and some cases of impaired wound healing (Gauthier and Sears, 1999; Bearzi, 2000; Cantor et al., 2010; Kiszka et al., 2010; Tezanos-Pinto and Baker, 2012; Noren and Mocklin, 2012). Alternatively, stranded cetaceans provide another valuable source to acquire significant knowledge of these marine mammals (Arbelo et al., 2013; Díaz-Delgado et al., 2018). Thereby, their carcasses represent a directly and no-limited access to take representative samples as well as collect skin biopsies and, therefore, evaluate diseases that may affect this organ without the risk of compromising the well-being of individuals. Correspondingly, most of the CePV reports attained by the best scientific evidence available have been made thanks to this considerable option, having this skin disease been identified worldwide with confirmed reports in the Atlantic sea (Geraci et al., 1979; Fiorito et al., 2015), Pacific oceans (Van Bressem et al., 1993; Van Bressem and Van Waerebeek, 1996; Bracht et al., 2006) and the North Sea (Blacklaws et al., 2013), affecting a broad range of cetacean species. Its geographic distribution is such that the emergence of this skin disease has also been proven in cetaceans under human care, concretely in common bottlenose dolphins (*Tursiops truncatus*) (Ridgway, 1984; Van Bressem et al., 2017; St Leger et al., 2018).

In relation to the latter, due to the growing social concern of the maintenance of these marine mammals under housed conditions, there has been a surge in the study of cetaceans' welfare in these institutions (Brando et al., 2018; Clegg et al., 2021; Lauderdale et al., 2021). Nowadays, this concept has become a priority for modern zoos, despite much of the work in the field of animal welfare has been performed in farm animals (Salas et al., 2018). However, the methods and approaches used in assessing farm animal welfare have been adapted and applied to the measurement of welfare in animals of other domains, including zoos (Clegg et al., 2015). Animal welfare has a complex nature, being composed by multidisciplinary factors such as physical and physiological health, emotional and behavior status (Broom, 1991; Carenzi and Verga, 2009; Manteca, 2012). Due to its heterogenicity, the study of animal welfare is challenging, leading most investigations to focus in one of the above-mentioned aspects or in the combined use of several qualitative variables which could be "resource" (environmental aspects) or "animal" based (behavioral, physical, and physiological aspects) (Clegg et al., 2017; von Fersen et al., 2018; Wolfensohn et al., 2018).

Correspondingly, the common approach to appraise animals' welfare in zoos is likely to perform qualitative assessments through animal-based indicators which relate to

physical health (Manteca et al., 2016; Whitham et al., 2017). In this manner, the prevalence and incidence of diseases and injuries has recently been proposed as a plausible indicator of health in relation to welfare in cetaceans (Clegg et al., 2015). Accordingly, poxvirus skin lesions, apart from having been considered as a potential health indicator, it may also behave as a variable to assess welfare, serving to identify possible unbalances in both the environment and host's health that may be related to its emergence. Hence, the easy accessibility of cetaceans in managed care may provide the knowledge, skills, and resources to understand the host-pathogen dynamics of cetacean poxvirus and their effect on cetaceans' health and welfare. Additionally, it's study under these conditions would also enable to extrapolate reachable findings from housed cetaceans to free-ranging populations.

Despite the above-stated advantages, little have been reported regarding CePV in cetaceans under human care in contrast to the ones in the wild (Figure 2). Possible reasons for this could be related to the previous mentioned evasiveness of skin biopsies. Under housed conditions, this technique may be considered a long-lasting manipulation that could entail physical restraint in addition to the fact that it is highly likely that the maintenance of individuals' well-being during the entire procedure could not be assured. In the event, employing this skin sampling technique to repeatedly assess skin diseases in cetaceans under human care could arise ethical awareness, especially when there are other less invasive sampling methods that could be used to try to determine their effectiveness in the subsequent diagnosis of the disease (Amos et al., 1992; Valsecchi et al., 1998; Gendron and Mesnick, 2001; Wang and Maibach, 2011).

Figure 2. Tattoo skin lesion on the peduncle from a bottlenose dolphin (*Tursiops truncatus*) under human care at Lanzarote Rancho Texas Park, Canary Islands, Spain. Image provided from the Animal Health and Food Safety Institute (IUSA), University of Las Palmas de Gran Canaria (IUSA).

Accordingly, the present research was developed to broaden the knowledge, to the extent possible, of the pathogenesis and epidemiology of cetacean poxvirus and other possible pathogens that may be associated with tattoo-like lesions and in a variety of different skin disorders from stranded cetaceans along the Canary coasts as well as from common bottlenose dolphins held in two facilities in the Canary Islands. Therefore, its specific objectives were:

1. To develop and probe the feasibility of a non-invasive skin sampling device to detect cetacean poxvirus by comparing with skin biopsies in a pilot study performed in stranded cetaceans.
2. To apply this non-invasive skin sampling technique in cetaceans held in zoos and aquariums to sample tattoo-like lesions without compromising their well-being, serving as a rapid sampling procedure, adaptable to caretakers and trainers from those institutions, and as an alternative device to skin biopsies.
3. To employ PCR techniques as sustainable diagnostic methods to determine associated pathogens in tattoo-like lesions and other skin disorders, as well as contribute to widening the limited genomic information of cetacean poxvirus.
4. To histopathologically characterize specific skin lesions and correlate them with macroscopic and molecular findings of selected pathogens (poxvirus, herpesvirus, and morbillivirus).

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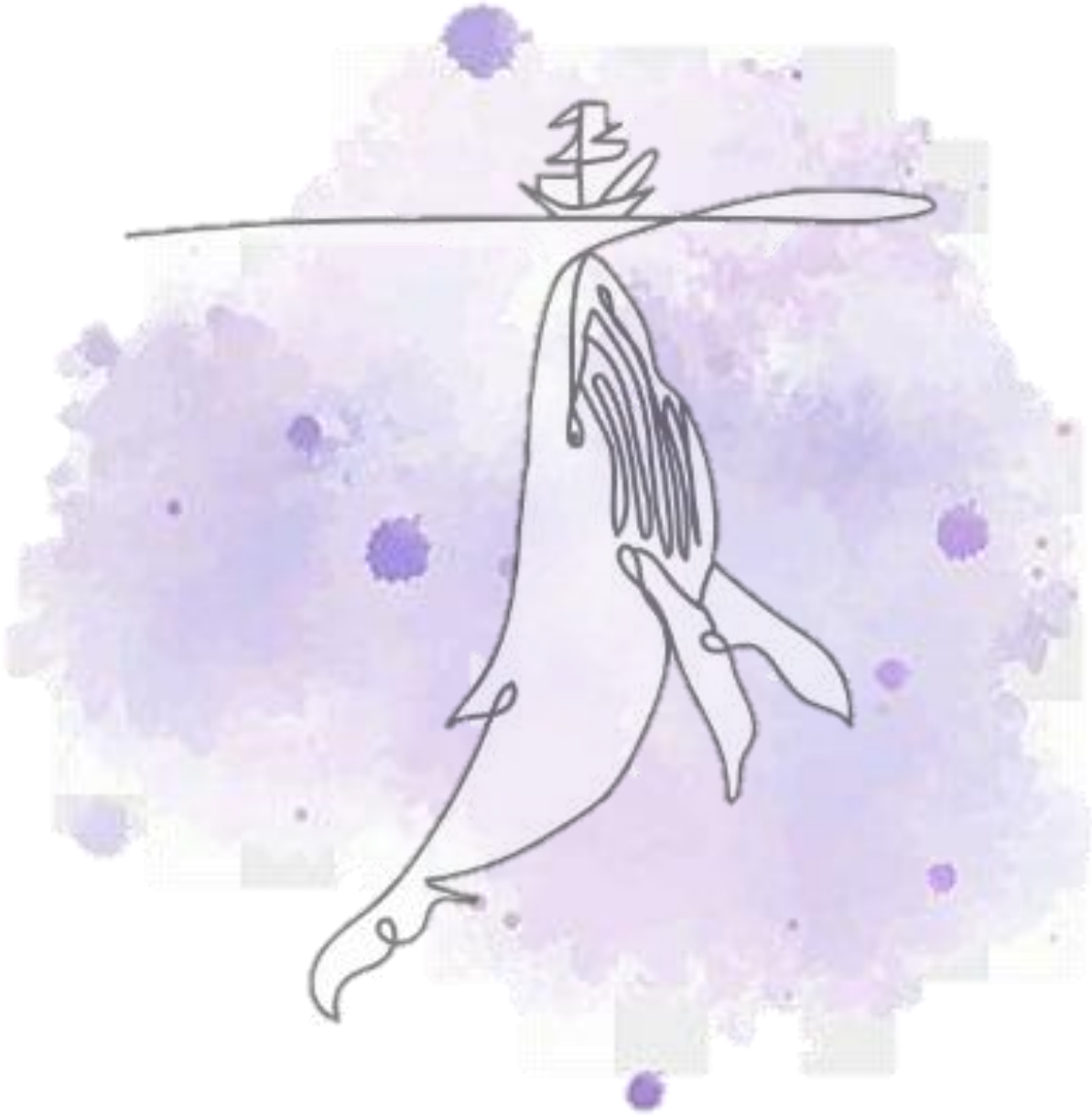
2.

SUMMARY OF PAPERS



To achieve the objectives of the present doctoral thesis, the following research which were embodied in three publications indexed in the Journal of Citation Reports, were performed:

- **FIRST PUBLICATION: THE VALIDATION OF A NON-INVASIVE SKIN SAMPLING DEVICE FOR DETECTING CETACEAN POXVIRUS.**

Most studies related to cetacean poxvirus consist of photographic surveys with visual identification of tattoo-lesions, and the presupposition that their emergence is due to the infection of this pathogen. Hence, the lack of infallible diagnostic methods to corroborate the presence of the virus in those lesions might result in inconsistent conjectures. For this purpose, the collection of skin biopsies from tattoo-like lesions and the subsequent molecular analysis is essential, which is the diagnostic method of choice in case of confirming cetacean poxvirus infection. Nevertheless, when dealing with cetaceans under human care, alternative sampling techniques rather than skin biopsies should be performed in order to enhance best handling procedures and contemplate ethical awareness, fulfilling the collection in a non-invasively manner. However, the uncertainty if feasible results in the further isolation of the pathogen could be achieved when employing other skin collection methods than skin biopsies may prompt.

Thus, this research consisted of a pilot study performed on twelve tattoo-like lesions of two stranded cetaceans on the Canary coast which were collected both with biopsies and cytology cell samplers (CCS), in order to compare the reliability of this latter device aiming to be further reproducible in cetaceans under human care as an alternative non-invasive skin sampling method. For this purpose, two different genomic extraction procedures were carried out (DNA Tissue Kit S™ (QuickGene, Kurabo, Japan) and DNeasy™ Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA)) with all the collected samples with the objective of associating which of them gives better results when comparing the sample collection method employed. Molecular detection of cetacean poxvirus was performed through a real-time PCR. As a result, 91.7% and 83.3% rates of positivity were obtained with biopsies and CCS through Quickgene, respectively, compared to the rate of 100% using CCS with Qiagen. Accordingly, CCS are a reliable non-invasive sampling device to obtain sufficient genetic material to be analysed for cetacean poxvirus in tattoo skin lesions in cetaceans under human care.

- **SECOND PUBLICATION: TOWARDS UNDERSTANDING HOST-PATHOGEN DYNAMICS OF CETACEAN POXVIRUS: ATTAINABLE APPROACH THROUGH THE APPLICATION OF A REPETITIVE NON-INVASIVE SKIN SAMPLING IN BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*) UNDER HUMAN CARE**

Zoos and aquariums, within their main three principles (conservation, education, and research) seek to maintain cetaceans under human care in their best health and welfare standards. To this end, besides being constantly reinventing, improving, and ensuring up-to-date health assessments, these institutions provide a significant opportunity to develop scientific evidence-based investigations to achieve a better approach to understanding cetaceans' health. Therewith physical status of cetaceans has been proposed to be an easy reachable animal-based resource to evaluate the overall health status of this marine mammals.

Hence, this study attempted to appraise skin diseases on cetaceans under human care, concretely Tattoo Skin Disease (TSD), through the improvement of skin sampling techniques with a non-invasive device, serving as an innovative approach to enhance animal management and handling. Thus, over the year 2019, a repetitive collection of sloughed skin with cytology cell samplers (CCS) from both tattoo-lesions and apparently healthy skin from 18 bottlenose dolphins housed in two facilities (Facility 1, FAC1 and Facility 2, FAC2) in the Canary Islands was carried out in order to detect cetacean poxvirus (CePV). This is the first report in which evaluation of the macroscopic progression of tattoo lesions with molecular corroboration of infection is performed over time. Moreover, the current survey has served to probe whether CCS consist in a practical non-invasive device being assertive in detecting CePV from sloughed skin. In the same manner, exceptional detection of the pathogen in healthy skin was also achieved not only in a social group where different individuals were showing the skin disease (FAC2), but also in a pod where macroscopical evidence of infection has not ever been reported (FAC1). Furthermore, the same sequence of CePV was derived from both tattoo lesions and skin samples without clinical evidence of the disease from both facilities and, furthermore, showed high homology to prior sequences obtained from free-ranging cetaceans throughout the North and South Atlantic Seas. The latter raises the question of whether this pathogen has persisted on zoos and aquariums through generations since the introduction of original wild-caught individuals around the 90s, being capable to produce latent infections, and whether progression of the disease may depend on environmental stimuli, viral load, or the good health/immunological status of the individual animals.

- **THID PUBLICATION: VIRAL SKIN DISEASES IN ODONTOCETE CETACEANS: GROSS, HISTOPATHOLOGICAL, AND MOLECULAR CHARACTERIZATION OF SELECTED PATHOGENS.**

Cetacean poxvirus (CePV) is widely known for inducing characteristic skin blemishes known as tattoo lesions, which show up as pinhole or ring-like forms with a stippled pattern on the centre, appearing flat or slightly raised, solitary or coalesced. Additionally, these lesions may both rapidly fade away to reappear again or persist over a long period of time. Due to its distinguishable skin manifestation, as far as it is concerned, no attempt to detect this pathogen in other skin lesions rather than “tattoos” has been reported. Neither it has been contemplated the possible consideration of the presence of concomitant pathogens in tattoo lesions which could influence their distinctive development over time.

In regard to the foregoing, this survey consists of a retrospective study where identification and macroscopical classification of eight different categories (tattoo-like (oval-shaped, coalesced, and serpiginous); black, white-fringed; pale; ulcerative; target-like; ring; and tortuous) of skin lesions with their respective descriptions from 55 skin lesions of 31 stranded cetaceans along the Canary coast between the years 2011 – 2021 were stablished. Subsequently, histopathological, and molecular analysis aiming to detect not only cetacean poxvirus but other emerging pathogens such as herpesvirus (HV) and cetacean morbillivirus (CeMV) were performed. Among results obtained, the most outstanding was that, molecularly, 47 skin lesions were positive (85.45%) to one or more of the viral agents tested in the present study, and only eight resulted negative (14.15%). Accordingly, coinfection of CePV and HV was achieved on nine lesions of eight cetaceans (16.36%), being this study the first report of comorbidity of both pathogens in cetaceans. Moreover, a feasible microscopical correspondence between CePV and HV positive lesions was achieved, enabling to histologically distinguish which pathogen was isolated in each lesion, or even in whatever lesion both viruses were detected. Besides, macroscopical and histological characterization of positive tattoo-like lesions and those displaying tortuous tracts was achieved.

3.

SCIENTIFIC PUBLICATIONS

animals

MDPI

Article

The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean Poxvirus

Simone Segura-Göthlin [1], Antonio Fernández [1,*], Manuel Arbelo [1], Idaira Felipe-Jiménez [1], Ana Colom-Rivero [1], Javier Almunia [2] and Eva Sierra [1]

[1] Veterinary Histology and Pathology, Atlantic Center for Cetacean Research, University Institute of Animal Health and Food Safety (IUSA), Veterinary School, University of Las Palmas de Gran Canaria (ULPGC), Trasmontaña, s/n, 35413 Las Palmas, Spain; siimone.andrea@gmail.com (S.S.-G.); manuel.arbelo@ulpgc.es (M.A.); idaira.fjvet@gmail.com (I.F.-J.); acolomrivero@gmail.com (A.C.-R.); eva.sierra@ulpgc.es (E.S.)

[2] Loro Parque Foundation, Avda. Loro Parque, s/n, 38400 Puerto de la Cruz, Spain; dir@loroparque-fundacion.org

* Correspondence: antonio.fernandez@ulpgc.es; Tel.: +34-928-451-095

Simple Summary: The current growing social awareness of animal welfare has led to the development of welfare indicators, which are effective tools for assessing each of the integrated aspects of this multidisciplinary issue. Hence, skin diseases have been suggested as potential general health indicators for use in cetaceans. Particularly cetacean poxvirus causes distinguishable hyperpigmented "ring" or "tattoo" lesions that affect cetaceans both in the wild and in managed facilities. However, most studies have analyzed these characteristic lesions through visual appraisal, while only a few have implemented diagnostic methods to corroborate the presence of the virus. To this end, skin biopsies are usually the sampling method selected, although they are considered to be an intrusive procedure. In this study, we analyzed sloughed skin sampled with cytology cell samplers (CCSs) in 12 tattoo-like lesions from two free-ranging cetaceans stranded in the Canary Islands. We employed two different DNA extraction methods and compared the effectiveness of the device with that of biopsies. All the lesions resulted positive for cetacean poxvirus, obtaining reliable data from the use of this device. Thus, CCS is considered to be a promising non-invasive tool for further assessing skin diseases in cetaceans, particularly those under human care, without affecting their welfare.

Abstract: Poxvirus-like lesions are widely used as a potential health indicator in cetaceans, although for this application, corroboration of Poxvirus skin disease is imperative. Aiming to address skin biopsies intrusiveness, a preliminary investigation of a non-invasive skin sampling procedure to molecularly detect CePV-1 in 12 tattoo-like-lesions from two free-ranging stranded cetaceans in the Canary Islands was performed. Skin lesions were brushed with cytology cell samplers (CCSs) and placed into 1.5 mL microcentrifuge tubes with 1 mL of RNA*later*™ Stabilization Solution. For factual comparisons, DNA extractions from sloughed skin obtained with CCS and biopsies from the same lesions were accomplished with DNA Tissue Kit S™ (QuickGene, Kurabo, Japan). Moreover, a second DNA extraction from sloughed skin with DNeasy™ Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) was performed to ascertain kit suitability for CCS. Molecular detection of CePV-1 was performed through a real-time PCR. As a result, a 91.7% and 83.3% rates of positivity were obtained with biopsies and CCS through Quickgene, respectively, compared to the rate of 100% using CCS with Qiagen. Accordingly, CCS is a reliable non-invasive sampling device to obtain sufficient genetic material to be analyzed for CePV-1 in tattoo-skin-lesions as well as for other purposes in cetaceans under human care.

Keywords: cetacean poxvirus; skin lesions; health indicator; welfare; biopsy; cytology cell sampler; DNA extraction; PCR; cetaceans

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1. Introduction

Cetaceans' skin is considered a multidimensional feature that can provide a wealth of information, forming the basis of research in a wide number of studies covering a broad range of scientific branches [1–8]. Hence, this tissue has been used for long-term health assessments, enabling us to gain a closer look at the health status of marine mammals and aquatic ecosystems [9–12]. For instance, skin diseases have been suggested to be triggered by exposing both free-ranging and human-managed cetaceans to continuous aberrant conditions, resulting in compromised immune system function and a consequent increase in susceptibility to disease [13–16].

Cetacean poxvirus (CePV) is one of the most widely reported skin diseases [17–21]. Recently, it has been classified into two groups: cetacean poxvirus 1 (CePV-1), which affects both free-ranging and human-managed odontocetes, and cetacean poxvirus 2 (CePV-2), which infects mysticetes [22,23]. In cetaceans, this cutaneous disease displays characteristic lesions which are recognized as hyperpigmented "ring" or "tattoo" lesions, with the latter being referred to as tattoo skin disease (TSD) [20]. Regarding the unanimous consensus that clinical signs of disease can be indicative of compromised health, these distinguishable skin manifestations have been considered as a potential general health indicator in cetaceans [13,24,25]. Despite being broadly described, this viral skin disorder is still unassigned within the *Chordopoxvirinae* subfamily due to the limited genomic information on it. One of the main reasons for this is that CePV has mainly been identified through visual appraisal [25–28], with few studies having used diagnostic assays to correctly detect and therefore determine the presence of this pathogen in poxvirus-like lesions in cetaceans [22,24,29–31].

The detection of CePV in tattoo-like lesions in cetaceans under human care is necessary in order for these lesions to be applied as an "animal-based" health indicator [32–35]. As most research fields in which skin is the focus of study, skin biopsies are the method of choice to either molecularly or histologically diagnose skin diseases in cetaceans in managed facilities [36–41]. Nevertheless, the increasing awareness of welfare in cetaceans has prompted attempts to develop dynamic methodologies for safe handling and sampling with the aim of minimizing the risk of compromising their well-being. As with wild individuals, in managed facilities some researchers have highlighted the fast turnover time of cetaceans' skin [42–46], proposing the collection of sloughed skin of animals' bodies as an advantageous non-invasive method that could potentially be an alternative to biopsies [5,47,48]. Notwithstanding the aforementioned points, research on the use of these emerging non-intrusive skin sampling techniques in cetaceans under human care is still scarce, with their effectiveness having been poorly explored in research studies.

Correspondingly, as managed facilities are actively committed to the advancement of scientific research while maintaining ethical responsibility, efforts to create innovative sampling methodologies and improve the standards of practice during these procedures should be encouraged [49–51]. Thus, stranded cetaceans could plausibly be used in model studies, providing an opportunity to perform preliminary investigations [52–54]. Additionally, their use would enable protocol adjustments to resolve possible misgivings and achieve feasible results that could be reproduced in cetaceans under human care.

Accordingly, the aim of the present study is to validate a potential non-invasive skin sampling device using sloughed skin to molecularly detect cetacean poxvirus (CePV) in tattoo-like lesions by comparing its sensitivity and effectiveness with that of skin biopsies obtained from stranded cetaceans in the Canary Islands.

2. Materials and Methods

On 21 February 2021, a juvenile male common bottlenose dolphin (*Tursiops truncatus*) (Case 1), 240 cm in length, was found stranded and dead at Abades, Arico, Tenerife, Canary Islands, Spain (28°09′00″ N, 16°25′00″ W). On 17 April 2021, a juvenile female Atlantic spotted dolphin (*Stenella frontalis*) (Case 2), 150 cm in length, was found stranded and dead at Playa San Juan, Guía de Isora, Tenerife, Canary Islands, Spain (28°10′47″ N,

16°48′45″ W). Based on their anatomic parameters, both animals showed a moderate body condition [55] and the carcasses were in a good state of preservation (code 2/5) [56–59]. Due to their exceptional states of preservation, neither the refrigeration nor freezing of either of the animals were required prior to their necropsies. Thus, standardized necropsies [60] were performed on each dolphin the day after they were found. Throughout the external examination during necropsies, several skin lesions affecting the rostral and lateral areas of both animals were observed. As a result, two lesions from Case 1 and ten lesions from Case 2 were described, photographed, and measured before their later collection. Each skin lesion from both animals was split to retain a portion at −80 °C, while the remainder was first sampled with a sterile cytology cell sampler (CCS) (Deltalab, Barcelona, Spain) and later correctly identified and preserved at the same temperature as the other portion. The skin sampling procedure using these CCSs consisted of gently brushing the surface of the lesions to obtain sloughed epidermis, which adhered into the bristles of the brush. Then, all the CCSs were placed into 1.5 mL sterile RNAse- and DNAse-free microcentrifuge tubes with a safe lock (Thermofisher Scientific, Madrid, Spain), in which 1 mL of RNA*later*™ Stabilization Solution (Thermofisher Scientific, Madrid, Spain) had previously been added. Subsequently, the bristles of the CCS stayed embedded in the RNA*later*™, while the plastic stems were cut to the level of the microcentrifuge tubes' tops with a pair of scissors to allow the closure of the vials, using the safe lock to avoid unexpected openings (Figure 1). Due to the genomic stabilization capacity of the *RNAlater*™ solution, microcentrifuge tubes were stored at room temperature until their subsequent molecular analysis, which was performed within 1 working week [61].

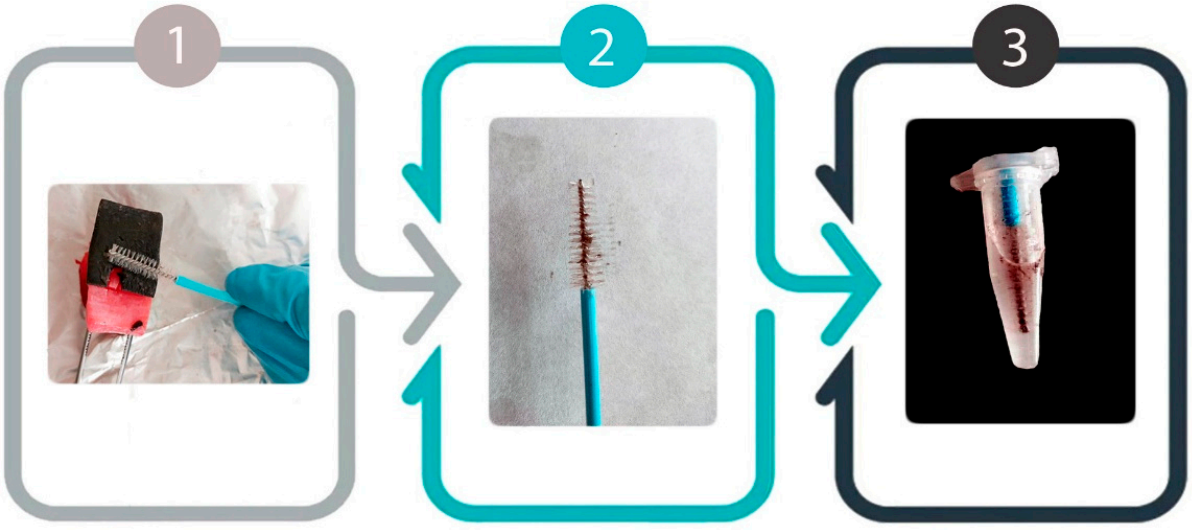

Figure 1. Workflow illustration of the cytology cell sampler skin sampling methodology.

After accomplishing the procedure previously explained, the rest of both necropsies were performed by sampling and collecting representative tissues of all the major organs and lesions for subsequent analyses in order to proximate the most plausible cause of death/stranding, as routinely performed [58,59]. Hence, all samples were stored in a 10% neutral buffered formalin fixative solution for histologic and immunohistochemical analysis, whilst few of them were preserved at −80 °C until processing for biomolecular studies.

Approximately 0.5 g of each fresh-frozen skin samples from both animals was mechanically macerated in lysis buffer and subsequently centrifuged, later progressing to simultaneous DNA/RNA extraction using the DNA Tissue Kit S™ (QuickGene, Kurabo, Japan). Considering that an initial sample of ≤0.5 g is required to correctly perform genomic extraction with this method, some modifications in the manufacturer protocol were necessary in order to accurately extract the DNA/RNA from the fresh skin samples collected with CCS. They were first agitated using a vortex for 15 s at maximum speed

to ensure the detachment and mixture of a great part of the epidermal crust adhered among the bristles into the 1 mL RNA*later*™ solution. After this, the tips of the CCS were removed, preserving the acquired RNA*later*–sloughed skin mixture in the vials. With the aim of obtaining an approximate amount of 5000 µL of macerates from each sample, some adaptations in the proportions of the components were made. Therefore, instead of adding 4500 µL of 0.1% diethylpyrocarbonate (DEPC)-treated water and 500 µL of 1× lysis buffer as accomplished with biopsy samples, 3600 µL and 400 µL from each component, respectively, were applied apart from the 1000 µL RNA*later*–sloughed skin mixture. Finally, all macerates were centrifuged (2500 rpm for 15′ at 4 °C) and supernatants were collected to continue with their genomic extraction. DNA/RNA extraction was achieved from each macerated sample (N = 24) in a QuickGene Mini 80 nucleic acid isolation machine (QuickGene, Kurabo, Japan) according to the manufacturer's instructions with some modifications: an RNA carrier (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA) was added during the lysis step, as previously indicated [62].

The molecular detection of CePV-1 was performed using a 1-step real-time polymerase chain (q-PCR) method to amplify a conserved region (150 bp) of the DNA polymerase gene by using the degenerate primer sets designed by Sacristán et al. [63] (Odontopox-F: 5′-CARGAAATMAAAAAGAARTTTCCATC-3′, and Odontopox-R: 5′-ACGTTCTGTTAARA AYCGTCTTAGTA-3′). The thermocycler profile was set for initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles, each compromised of a denaturation step at 95 °C for 15 s, an annealing step at 60 °C for 30 s, and an elongation step at 72 °C for 30 s. The final cycle was composed of an extended elongation, which was performed at 72 °C for 7 min [29]. A melting curve step was added at the end of the reaction. The thermal cycler employed was a MiniOpticon™ Real-Time PCR System (Bio-Rad Laboratories, Irvine, CA, USA). Adequate non-template negative controls (nuclease-free water) for both extraction and amplification as well as extraction-positive and amplification-positive controls previously confirmed by our group were included.

The PCR products from positive lesions were purified using a commercial kit (Real Clean Spin kit 50 Test-REAL), and then sequenced using Sanger DNA sequencing (Secugen S.L., Madrid, Spain). The amplicon identities were confirmed with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi/ (accessed on 4 June 2021)).

In order to compare the effectiveness of the DNA extraction from the skin samples collected with CCS using the QuickGen kit method, a second extraction using DNeasy™ Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) was undertaken. In this instance, it was necessary to unfreeze each of the halves which had previously been scraped with CCS from the 12 skin samples collected from Cases 1 and 2. Hence, the same skin sampling procedure using CCS explained above was performed repeatedly. The CePV-1 positive control was a 0.025 g biopsy that had previously been confirmed by our group using the real-time PCR method [63] and sequencing amplicons (unpublished sequencing results) previously described. Once 1 mL RNA*later*–epidermis mixtures were obtained, they were subsequently subjected to a high centrifugation speed for 5 min (14,000 rpm). On some occasions, this step had to be repeated as many times as necessary to obtain enough pellet. Consequently, approximately 0.025 g of skin pellet precipitation was collected from each sample by removing practically all the supernatant. To verify that the maximum sample weight specified by the manufacturer's instructions for correctly performing the DNA extraction had not been exceeded, all the vials were weighted. Thus, precise weights of samples were acquired by subtracting the weight of an empty vial (≈1.078 g). Afterwards, all samples were ready for the DNA extraction to proceed following the manufacturer's instructions. The importance of incubating the biopsy CePV-1 positive control, which had previously been cut into small pieces, for at least 30 min with continuous 15 s high-speed vortexing every 5 min during incubation for complete lysing must be noted. Upon the completion of the extraction, DNA products were tested with the same real-time protocol as that mentioned above (see Appendix A for more details).

3. Results

3.1. Macroscopic Findings

3.1.1. Case 1

In total, Case 1 (Figure 2) showed two lesions that could be attributable to CePV. Of both lesions, the most remarkable was a 5 × 3.5 cm serpiginous and stippled light grey tattoo-like lesion located on the ventral right corner of the oral cavity (Figure 2A). The other lesion (Figure 2B) showed an oval and depressed shape, which was observed on the melon of the common bottlenose dolphin.

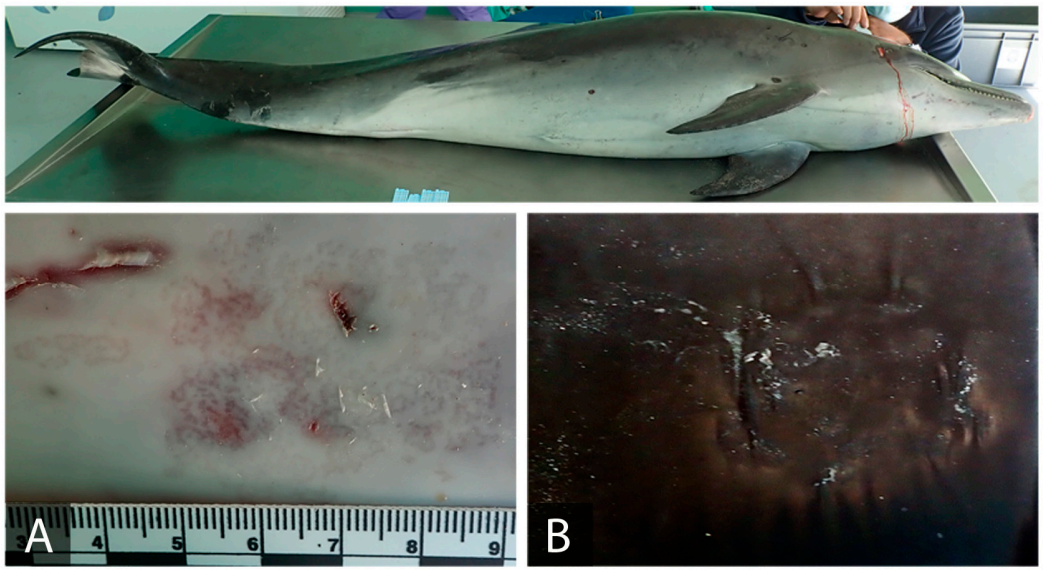

Figure 2. Gross lesions compatible with CePV in bottlenose dolphin, Case 1. Right lateral view. (**A**) Irregular, stippled, and serpiginous grey lesion (5 × 3.5 cm) located ventral to the right corner of the oral cavity. (**B**) Oval and depressed lesion (2.5 × 2 cm) on the right lateral side of the melon.

3.1.2. Case 2

Just like Case 1, Case 2 (Figure 3) presented compatible CePV lesions. In a multifocally manner, tattoo-like lesions with different evolution stages were randomly distributed and affected many areas of the skin. Three of them (Figure 3A–C) were the characteristic persisting ring lesions, delimited with black edges and showing a black and stippled pattern at the center. One of these lesions (Figure 3C) showed blistering across half of its center. Another two lesions (Figure 3D,E) were observed on the tip and melon of the dolphin, respectively. They were lighter in color and featured a barely visible black margin, corresponding to the lesions in the healing process. On one of the flanks of the spotted dolphin, a ring lesion that was black in color with pale edges was observed (Figure 3F). Close to it, a lesion that appeared very similar to this last one, apart from its pale, irregular, and raised center, was observed (Figure 3H). The lesions observed at the ventral part of the animal (Figure 3G,I) were irregular, light grey, and blurred, being hardly perceptible and without delimiting margins. On the peduncle, there was a remarkably large lesion affecting almost all the entire length (Figure 3J). This lesion was irregular in shape and black, and featured a pale grey pin-hole pattern along its center.

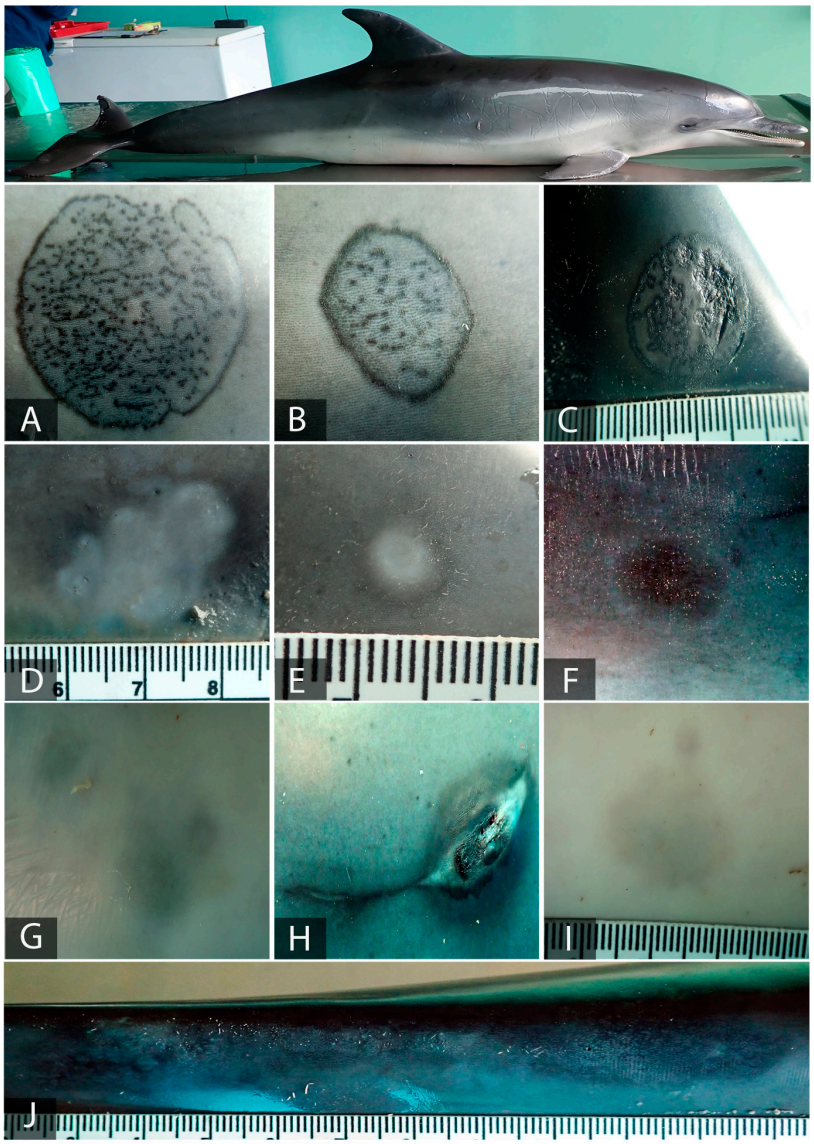

Figure 3. Gross lesions compatible with CePV in Atlantic spotted dolphin, Case 2. Right lateral view. (**A**) Ring lesion with a black edge and stippled pattern center (3 × 2.3 cm) on the right side of the melon. (**B**) Ring lesion with a black edge and stippled pattern center (1 × 0.7 cm) located on the right side of the melon. (**C**) Oval lesion presenting both margin and inner ping-hole pattern slightly raised with half of the center blistered (1.8 × 1.3 cm), located on the right side of the dorsal fin. (**D**) Irregular pale and coalesced wound with a barely visible dark edge (2.3 × 1.2 cm) located on the right dorsolateral superior hemimaxilla. (**E**) An oval lesion with a pale center and blurred margin (0.6 × 0.3 cm) situated on the right dorsal part of the tip. (**F**) Oval dark lesion with pale margin (1.5 × 1 cm) located on the right side of the animal. (**G**) A blurred and irregular grey lesion on the rostroventral part. (**H**) An oval dark lesion with a pale, raised, and irregular center (1.6 × 1.3 cm) on the right lateral side. (**I**) A blurred hardly visible grey lesion (1.8 × 1.5 cm) on the ventral part of the animal. (**J**) A large and irregular dark lesion with a greyish pin-hole pattern across the entire center located on the dorsal part of the peduncle.

3.2. Molecular Findings

The results of the molecular findings are compiled in Table 1. Of the 12 cutaneous lesions sampled using biopsies taken from both individuals, which were previously submitted for Quickgene DNA/RNA extraction, 11 were positive for CePV-1. More specifically, from Case 1, both lesions were positive; meanwhile, in Case 2, of the 10 lesions tested,

nine were positive. Only one lesion (Figure 3J) presented an abnormal amplification curve with a RT-PCR cycle threshold value (Ct) of 11.66 without melting temperature. For the purpose of confirming this lesion as negative to CePV-1 and to prove that the PCR product was neither too concentrated nor overloaded with inhibitors leading to incorrect PCR interpretations, it was diluted into 10-fold serial dilutions up to 10^{-3}. In this way, we sought to stablish a better sensitivity and quantification dynamic range. However, the real-time PCR detected neither of the dilutions of the PCR product from this lesion.

Table 1. Molecular results from tissue and cytology cell sampler sampling methods using both the DNA Tissue Kit S™ (QuickGene, Kurabo, Japan) and the DNeasy™ Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) on skin lesions from a bottlenose dolphin and an Atlantic spotted dolphin stranded on the Tenerife coasts, Canary Islands, Spain, 2021.

Case	Lesion	Tissue		Cytology Cell Sampler			
		QuickGene		QuickGene		Qiagen	
		CePV	Ct	CePV	Ct	CePV	Ct
1	A	+	23.65	+	25.46	+	20.85
	B	+	31.80	+	27.93	+	26.70
2	A	+	15.65	+	17.25	+	17.14
	B	+	18.08	+	20.80	+	19.58
	C	+	16.42	+	19.04	+	15.17
	D	+	33.63	+	34.45	+	34.27
	E	+	25.02	+	33.55	+	34.74
	F	+	33.44	+	34.31	+	35.09
	G	+	31.79	−	n/a	+	37.10
	H	+	35.37	−	n/a	+	35.66
	I	+	28.49	+	33.33	+	31.97
	J	−	n/a	+	33.20	+	31.88

Notes: CePV-1, cetacean poxvirus; Ct, cycle threshold; Qiagen, DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA); QuickGene, DNA Tissue Kit S (QuickGene, Kurabo, Japan); +, positive; −, negative; n/a, not applicable.

Regarding the samples collected with sterile CCS from these 12 lesions, different results were obtained depending on which genomic extraction method was used. Hence, with Quickgene, 10 lesions from both cases were found to be CePV-1 positive: both lesions from Case 1 (Figure 2A,B) and eight lesions from Case 2 (Figure 3A–F,I,J). Comparing these results with those obtained with tissue sampling, it can be observed that, in both sampling methods, the same lesions from Case 1 were found to be positive for cetacean poxvirus. Nevertheless, the same outcome was not observed in Case 2, in which a lesion that was found to be negative when sampled with a biopsy (Figure 3J) was found to be positive when sampling using CCS. However, using this latter sampling method, two other lesions which were found to be positive for CePV-1 when collected using a biopsy (Figure 3G,H) were not detected. Conversely, through Qiagen, all lesions from Case 2 were found to be positive for poxvirus, in addition to the other two lesions from Case 1. Therefore, making a general comparation from these results with the other obtained by employing a different genomic extraction method, we observed that using the same sampling procedure (CCS), the two lesions that were not detected (Figure 3G,H) were both found to be positive with Qiagen. Moreover, with this last genomic extraction kit, the negative tissue sample from Case 2 was also found to be positive when CCS was employed. Thus, from a broad-based assessment, we could observe that with Quickgene, a slightly better sensitivity was acquired when samples were collected with biopsy rather than with CCS. Yet, an improvement on these results was gained when applying both the CCS sampling method and the Qiagen extraction kit.

Comparing the Ct values from positive lesions between both different sample collections and genomic extraction methods a remarkable range of the Ct values was observed, with 15.17 and 37.10 being the minimum and the maximum values, respectively. Consider-

ing the theoretical correlation in which it was established that low Ct values correspond to a high viral loads and vice versa, it is observed that the lesion 3A from Case 2 presented the maximum viral load in both sampling techniques and extraction methods. In addition, the same sigmoidal correlation was observed between the lesions that had the lowest viral load extracted with Quickgene. Thus, lesion 3H, which was sampled by biopsy, had a Ct value of 35.37, with it being undetected when using the cytology cell sampler. However, these results did not concur with the ones obtained with Qiagen, with lesion 3G being the one which presented the least viral load, with a Ct value of 37.10.

The sequence similarity searching from the DNA polymerase sequences of CePV-1 obtained from all the positive lesions of both cases in this study was performed with BLAST. We could identify that, in both cases, the sequences revealed high percentage homologies of 100%, 99%, and 98% with the already uploaded nucleotides sequences under the GenBank accession numbers of MF458199, KU726612, and MH005249, respectively.

4. Discussion

4.1. Sampling Methods and DNA Extraction Protocols

In the present study, the molecular detection of cetacean poxvirus from two free-ranging cetaceans stranded in the Canary Islands was achieved, with different results being obtaining depending on the sampling method used and the genomic extraction kit employed.

Aiming to reproduce and extrapolate the respective sampling procedures used for cetaceans under human care, skin samples were attained in fresh conditions. The use of sterile CCS enabled us to obtain an acceptable quantity of sloughed skin from all poxvirus-like lesions for posterior genomic extractions. Macroscopically, the load of sloughed skin that adhered to the bristles of CCS was determined by the size of the lesions, with it being possible to gain more epidermal crust from larger samples than was possible from smaller ones. This resulted in it being easier to obtain epidermal crusts from lesions with a larger volume due to it being easier to rub their surfaces than ones with a smaller size, with it being necessary in the latter to scrape the outer layer more times to gather sufficient desquamating epidermal debris. Accordingly, the time required to finally acquire sloughed skin was found to be approximately 1 min in all attempts. Nevertheless, as was recently reported by Bechshoft et al. [5], enough skin cannot always be obtained from scraping alongside the flanks of bottlenose dolphins in managed care when using a rubberized scraper. This may be due to the high metabolic and mitotic activity which affected skin undergoes, leading to a continuous removal of epidermis, in contrast to healthy skin [17,18]. In either case, the variation in the quantity of sloughed skin obtained between each sample did not lead to further complications for the DNA extraction, as each of the protocols was standardized.

Since biopsy is the current method of choice for collecting skin samples, we decided to compare the genomic yield gained from this sampling method with that obtained through CCS by using a DNA extraction kit that was specifically suitable for use with tissue samples [64]. Therefore, through Quickgene, we attempted to contrast the reliability and effectiveness of both sampling procedures in order to gain enough genetic material from the skin lesions.

Furthermore, Qiagen was used for a second DNA extraction from sloughed skin obtained from the same poxvirus-like lesions. According to the manufacturer's protocol, Qiagen is suitable for purifying DNA from very small amounts of starting material, ensuring high-quality yields from nonstandard samples and considering 0.025 g as the maximum weight [65]. Therefore, the purpose of this second genomic extraction was to corroborate the point mentioned above, comparing the genome extraction from the sloughed skin that was collected via CCS through both kits. Thus, this study not only attempted to show which of the two sampling methods obtained more sensitive results, but also attempted to ascertain which of the genomic extraction protocols is more appropriate for use with the proposed non-invasive sampling method. In contrast to the first extraction, which

was carried out through Quickgene, the undeliberate unfreezing of skin lesions from both animals had to be conducted to repeat the skin sampling procedure with CCS. This feature is recognized to have an undesirable impact on the quality of DNA preservation, apart from not serving as a standard operating procedure if it is intended to be extrapolated in cetaceans kept in managed facilities. Despite this, the DNA extraction was carried out while considering the latter facts regarding the further interpretation of the molecular results.

In both the CSS sampling procedures, the sloughed skin was embedded in RNAlater™ Stabilization Solution. The purpose of the use of this reagent is that it can serve as a transport medium in situations in which samples cannot be immediately processed or frozen, as often happens in managed facilities, where samples are normally sent to external laboratories.

4.2. q-PCR Molecular Results from CePV-1 Positive Lesions

The visual diagnosis of TSD was confirmed in all the samples tested in the present study. Nevertheless, the results differed when using the different tissue sampling methods and DNA extraction kits.

Focusing on the juvenile bottlenose dolphin (Case 1), both lesions were found to be positive when employing both sampling and genomic DNA extraction protocols. Lesion 2A presented a typical serpiginous irregular pattern and was delimited with black borders. In the literature, these lesions are considered to represent the acute phase of infection [24]. On the other hand, the other lesion, 2B, would have been hard to detect if it were not for its depressed and oval-shaped appearance. This lesion is considered to be in an advanced stage of the infection [18]. Through Quickgene, it can be observed that both the biopsy and CCS sampling methods were effective for both lesions. However, it is evident that lesion 2A showed a higher viral load than lesion 2B, with the biopsy sample showing a better Ct value (23.65) than the sloughed sample (25.46). Nevertheless, both values indicate a considerable viral load when in terms of poxvirus infections. Thus, there is a correlation between the macroscopic findings, since 2A was considered to be in an initial stage and due to the viral load. Regarding the other positive lesion, 2B, it was also successfully extracted using both sampling methods. However, in this case it was the sloughed sample that presented a better Ct value (27.93) compared to the biopsy (31.80). In addition, these molecular results are also correlated with the advanced stage of the lesion. The Qiagen extractions of sloughed skin collected from both lesions gained good DNA genomic yields, to such an extent that each lesion presented even better Ct values than those found using the Quickgene extraction, with Ct values of 20.85 and 26.70, respectively.

In the Atlantic spotted dolphin, different evolution stages of 10 tattoo-like lesions were observed, coinciding with a wide range of Ct values. Macroscopically, the first three lesions (3A–C) were typical rounded lesions with a stippled pattern in the center, representing the early stage of the infection [24]. Lesions 3A and 3C were larger in size than 3B and also presented considerably more dark pinpoints at the center. Moreover, lesion 3C presented slightly raised margins and half of its center was blistered; both features could be used to identify acute phases of the infection [18]. Regarding the Quickgene biopsy genome extractions, the Ct values obtained from these three tattoo-like lesions were 15.65, 18.08, and 16.42, respectively, with these lesions having higher viral loads than all the others tested in the present study. The same pattern can be observed in the molecular results from the CCS, with Ct values of 17.25, 20.80, and 19.04. In this case, the biopsy samples gathered better genomic yields than the sloughed samples when using same extraction kit. Concerning the DNA extraction yield with Qiagen from the sloughed skin, very similar results are obtained. Regarding these first three lesions, in this case the biopsy samples gained better Ct values from 3A and 3B, with only the result for the third lesion, 3C, being improved with the use of Qiagen.

Within the other seven lesions from specimen 2, the variations in molecular values between the sampling methods through Quickgene were significant. At first glance, the lack of CePV amplification on the three lesions can be noticed. Regarding 3J, we were not able to amplify poxvirus DNA using the biopsy sample. Conversely, the same lesion was amplified

when using the CCS sampling technique. Our first impression was that the PCR product from the biopsy sample was overloaded, leading to amplification faults. However, once they were diluted into serial dilutions, none of them were found to be positive for CePV. These results might suggest that an inappropriate genomic DNA extraction procedure was used for this lesion, or that incorrect sampling was achieved due to selecting an area from the lesion without viral content. Whatever the case, the Ct value from the sampling of this lesion with CCS was 33.20, indicating a low viral load, a feature which might have also influenced the result obtained with the biopsy. The other two lesions from which DNA was extracted that did not have amplified poxvirus sequences were 3G and 3H. In this case, both lesions were sampled with CCS. This might have been due to their low viral loads (the samples presented values of 31.79 and 35.37 from biopsy sampling, respectively), meaning that they were undetected when collected from sloughed skin. On the other hand, and corroborating the above point, lesion 3G was barely visible macroscopically and featured a dark-grey area, appearing to be an almost healed tattoo-like lesion [33]. However, contrary to what might be expected, lesion 3H, which was compared to 3G in a prior evolution stage, presented a lower viral load than the other lesion. When analyzing the molecular results obtained through Qiagen for these three lesions, it is possible to reach more reasonable conclusions. The DNA extracted from sloughed skin from lesions 3G, 3H, and 3J using this kit were found to be positive for CePV-1. The Ct values obtained were 37.10, 35.66, and 31.88, respectively. In this case, an expected clear correlation between the low expression level of Ct values and macroscopical findings can be observed. Interestingly, the Ct results for lesion 3J were more favorable when using this genomic extraction protocol employing CCS as a sampling method. Accordingly, as has been reported, Ct values of above 35 in q-PCR are not considerable and should not be interpreted as marginally positive. However, the melting curves obtained from these PCR products were identical for all positive samples, and negative controls did not produce any product. Due to this, they were sequenced to confirm their specificity, leading to poxvirus DNA polymerase sequences being obtained.

The rest of the lesions (Figure 3D–F,I) were all in different stages of regression, with black margins being less evident or disappearing and the lesions becoming lighter in color [20]. The four of them presented low viral loads when using both genome DNA extractions, with slightly better Ct values being obtaining with the biopsy compared to with CCS through Quickgene. The other genomic extraction protocol obtained barely weakened molecular results compared with both sampling methods.

4.3. Validating Cytology Cell Samplers as a Reliable Non-Invasive Method to Sample Skin Lesions

In attempting to determine the effectiveness of the CCS, different percentages of positive results were obtained when comparing the sampling and genomic extraction methods. In this manner, considering that all 12 lesions were determined to be CePV-1 positive through Quickgene, the effectiveness of detecting this virus in skin lesions was 91.7% and 83.3% when using biopsy and CCS, respectively. From this, it can be deduced that sampling with skin biopsies has an 8.4% accuracy. Comparing the Ct values of both sampling methods when using Quickgene as a genome extraction kit, it is evident that the lesions sampled with CCS require more amplification cycles in order to cross the positivity threshold. This is reflected in the negative Ct values of the lesions sampled with CCS (Figure 3G,H). Accordingly, as mentioned before poxvirus lesions in healing stages with low viral loads might lead to CCS losing a certain amount of sensitivity. However, these results could be improved by the use of Qiagen with CCS, which had a 100% success rate.

Considering physical status as an important aspect of an animal's overall wellbeing, detecting cetacean poxvirus in tattoo-like lesions is important in order to correctly corroborate an animal's condition. Hence, such characteristic lesions could generally serve as an indicator of disease progression, thus correlating them with the health state of the animal. In cetaceans under human care, the presence of confirmed poxvirus lesions could potentially be used as a visual health parameter, especially when combined with the advantage

of applying CCS as a non-invasive skin sampling procedure which is unlikely to negatively interfere with the welfare of animals.

Having access to two specimens with positive poxvirus lesions was very significant in our quest to validate CCS as an effective viral skin sampling method. The exceptional states of preservation of the animals was crucial in developing the present skin sampling protocol in order to be used for cetaceans under human care. In addition, despite the limitations of the sample size, the fact that all 12 lesions were found to be positive for cetacean poxvirus through CCS was outstanding and reaffirms the need to prove their efficacy in cetaceans under managed care.

In summary, this pilot study on stranded animals has served as an opportunity to validate the use of sterile CCS for the diagnosis of poxvirus skin disease. The skin sampling procedure making use of sterile CCS can be considered to be a promising method for the detection of cetacean poxvirus, with accurate results for animals in managed care. Furthermore, we can additionally consider their implementation for sampling sufficient genetic material for other multiple areas of study, not limiting their applicability in poxvirus skin disease. Additionally, this leads to the idea that there should be a rigorous discussion as to whether biopsies are truly the best sampling method for detecting pathogenic microorganisms such as viruses in cetaceans under human care. This needs to be balanced against the potential stress and risk caused to the individual by the handling and sampling processes. However, further investigation is needed to address the uncertainties involved and ensure the potential of the use of this non-invasive method in cetaceans in managed facilities.

5. Conclusions

In the present study, we demonstrated the reliability of the use of CCS for the detection of cetacean poxvirus, comparing the results with those of biopsy samples. These findings will be highly significant for validating the further use of this device as a non-invasive method for assessing viral skin lesions in cetaceans under human care and carrying out visual health assessments.

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Appendix A

As figured in the handling protocol, the DNA Tissue Kit S^{TM} (QuickGene, Kurabo, Japan) corresponds to genomic extraction from 0.5 g of animal tissue sample, making it ideal for genomic DNA/RNA extraction from the 12 poxvirus-like lesions biopsy samples used in the present study. Thus, 0.5 g of tissue from each skin lesion was used as a maximum amount of starting material. Regarding the samples collected with CCS, despite it being practically impossible to obtain the same quantity of sloughed skin from every skin lesion, adaptations were made in terms of the proportions of the maceration components. This resulted in there being 12 macerates with a final volume of 5000 µL, consisting of

1000 µL of RNAlater mixed with sloughed skin, 3600 µL of 0.1% DEPC-treated water, and 400 µL of 1× lysis buffer. In this manner, samples collected with CCS presented the same volume and did not broadly vary.

During the genomic extraction with the DNeasy™ Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA), prior to the lysis step, once all samples were centrifugated it was noticed that some of the vials did not show sloughed skin precipitations. On some occasions, repeated high-speed centrifugations were needed in order to achieve the decantation of the desquamating particles for further 0.025 g obtention. Despite this, DNA extraction from all sloughed samples could be conducted without some constraints. However, during the lysis step, extra time was needed for the positive control (0.025 g skin biopsy of a positive CePV-1 lesion from a short-finned pilot whale) in order to finally complete the lysis. Despite the small sample size and the fact that it was cut into even smaller pieces, the firmness of this tissue required the use of 30 min of incubation at 56 °C with continuous vortexing. Indeed, this is one of the reasons why we decided to carry out the genomic extraction of biopsy samples with Quickgene.

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EDITED BY
Lusheng Xin,
Chinese Academy of Fishery Sciences, China

REVIEWED BY
Salvatore Siciliano,
Fundação Oswaldo Cruz (Fiocruz), Brazil
Cristiano Cocumelli,
Institute of Experimental Zooprophylactic of the Lazio and Tuscany Regions (IZSLT), Italy
Susan Smith,
Mystic Aquarium, United States

*CORRESPONDENCE
Antonio Fernández
antonio.fernandez@ulpgc.es

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Towards understanding host–pathogen dynamics of cetacean poxvirus: attainable approach through the application of a repetitive non-invasive skin sampling in bottlenose dolphins (*Tursiops truncatus*) under human care

Simone Segura-Göthlin[1], Antonio Fernández[1]*, Manuel Arbelo[1], Javier Almunia[2], Lorenzo von Fersen[3], Katrin Baumgartner[3], José Guerra Garcés[4], Aldo Gutiérrez Llanos[4], Idaira Felipe-Jiménez[1], Ana Colom-Rivero[1] and Eva Sierra[1]

[1]Veterinary Histology and Pathology, Atlantic Center for Cetacean Research, University Institute of Animal Health and Food Safety, Veterinary School, University of Las Palmas de Gran Canaria, Las Palmas, Spain, [2]Loro Parque Foundation, Puerto de la Cruz, Spain, [3]Zoo Nuremberg, Nuremberg, Germany, [4]Rancho Texas Lanzarote Park, Las Palmas, Spain

Nowadays, zoos and aquariums, along with the constant advancement of sociocultural moral values, are proactively committed to ensuring and safeguarding cetacean health standards. This entails developing new approaches to health assessments by embracing minimally invasive sampling methods and enhanced animal handling and management, among other aspects. Hence, in the present survey, to appraise skin diseases, the implementation of cytology cell samplers as a non-invasive skin sampling device on 18 bottlenose dolphins housed in two facilities in the Canary Islands during the months of April, October, and December 2019 was performed to isolate cetacean poxvirus in tattoo-like lesions through a real-time PCR-based method using the DNA polymerase gene. Samples were repeatedly collected over time from eleven tattoo-like lesions and from apparently healthy skin to serve as a control for all study animals. From a total of 55 skin samples, detection of the poxvirus was attained in 31 (56.36%); specifically, on 20 of 21 samples collected from tattoo-like lesions (95.23%) and on 11 of 34 samples acquired from apparently healthy skin (32.35%). Correspondingly, the current study constitutes the first report of the isolation of cetacean poxvirus in skin samples without macroscopical signs of tattoo lesions in cetaceans. Likewise, ten of the eleven dolphins that showed tattoo lesions housed in Facility 1 were positive for tattoo skin disease, while four dolphins held in Facility 2 were positive for cetacean poxvirus without ever showing clinical evidence of the disease. This raises the question of whether this pathogen can produce latent infections and whether progression of the disease may depend on environmental stimuli, viral

load, or the good health/immunological status of individual animals. Accordingly, further scientific research on cetaceans under human care could provide the knowledge, skills, and resources to understand the host–pathogen dynamics of cetacean poxviruses and their effect on cetaceans' health.

KEYWORDS

bottlenose dolphins, cetacean poxvirus, cytology cell sampler, health, PCR, skin lesions, under human care

1 Introduction

During the past few decades, growing social concern regarding cetacean well-being has been accompanied by a remarkable ethical awareness of their maintenance in public display facilities (Jiang et al., 2008; Brando et al., 2018; Makecha and Highfill, 2018; Miller et al., 2018). This has forced these establishments to undergo reinvention to adapt to the demands of a constant change in social moral values and culture (Fraser and Switzer, 2021; Miranda et al., 2022). By this means, current modern zoo and aquarium objectives are not only based on ensuring animal conservation but also on promoting public education and recreation and taking part in scientific investigation (Draper and Harris, 2012; Gross, 2015; EAAM, 2019; EAZA, 2019; Rose and Riley, 2022). What these four goals have in common is that, in order to attain them, cetaceans under human care must be provided with the best health conditions possible (Daoust et al., 2014; Brando et al., 2018; Samelius, 2018; Wolfensohn et al., 2018).

A common approach to assessing animal health in zoos is to perform qualitative assessments through animal-based resources that relate to physical health and/or the prevalence and incidence of diseases and injuries, among others (Broom, 1991; Lerner, 2008; Clegg et al., 2015; Salas and Manteca, 2016; Whitham et al., 2017). For instance, the body condition score, commonly used in farm animals, is beginning to be applied to bottlenose dolphins under human care with the aim of assessing their nutritional status and overall health (Wells et al., 2004; Clegg et al., 2015; Patterson, 2016; Castrillon and Nash, 2020). Another proposed physical bodily measure of health is the quantification of rake marks as an indirect measure of aggression in cetaceans. This has already been applied to other studies (Pettis et al., 2004; Scott et al., 2005; Clegg et al., 2015). Further, best animal management and handling practices, along with the fulfillment of ethical matters, go hand in hand with animal health assessments (Lauderdale et al., 2021a; Schilling et al., 2022). Thus, the goal of any investigation developed in those facilities might be conducted along with the enhancement of innovative approaches and/or the improvement of sampling techniques to assess their well-being through minimally- or non-invasive methods, usually aiming to avoid long-lasting manipulations of animals (Zemanova, 2020; Schilling et al., 2022). Hence, different methods that minimize impact while sampling marine mammals in managed care have been reported, including the use of exhaled breath condensate to examine respiratory-associated microbial microorganisms (Lima et al., 2012), rubberized scrapers to obtain sloughed skin to further determine skin cortisol concentrations (Bechshoft et al., 2015), or swabs to collect saliva (Rickert et al., 2022), among others.

Accordingly, a previous study (Segura-Göthlin et al., 2021) proved the feasibility of using cytology cell samplers (CCS) as a skin sampling method to detect cetacean poxvirus (CePV) on sloughed skin when compared to skin biopsies on stranded cetaceans. The results showed that slightly increased sensitivity to further molecular isolation of this pathogen was gained through sloughed skin collected with this device in contrast to biopsied samples. Thus, CCS could serve as a novel and reliable skin sampling technique for cetaceans under human care, with the view that it might be a potential alternative to avoid invasive and enduring manipulations as well as minimize the risk of affecting their well-being during the procedure. Although CePV is currently not known to be lethal (van Elk et al., 2000), it is thought to be a potential health indicator due to the relative ability to distinguish their characteristic skin blemishes known as "tattoo-like" lesions, which show gray, black, or yellowish color with an irregular stippled pattern (Powell et al., 2018; Rodrigues et al., 2020; Luciani et al., 2022) and because its clinical manifestation has been implied to be the reflection of long-term environmental pressures, making the animals more susceptible to disease (Van Bressem et al., 2009b; Bossart and Duignan, 2018; Koch et al., 2018). In line with the aforementioned and because of their distinctive macroscopical appearance, a significant extent of what has been reported regarding CePV consists of photographic surveys identifying tattoo-like lesions in free-ranging cetaceans, assuming their emergence is related to CePV infection (Van Bressem et al., 2003; Riggin and Maldini, 2010; Fury and Reif, 2012; Powell et al., 2020). However, despite being highly recognizable, it is important to diagnose CePV in those lesions using diagnostic-based methods to confirm tattoo skin disease (TSD) and avoid subjective assumptions.

Since its first description through transmission electron microscopy (TEM) about several decades ago (Flom and Houk, 1979; Geraci et al., 1979; Van Bressem et al., 1993; Van Bressem and Van Waerebeek, 1996), which is considered, together with PCR techniques, the key diagnostic methods for CePV identification (Blacklaws et al., 2013; Barnett et al., 2015; Luciani et al., 2022), poxvirus-like lesions have been reported worldwide (Van Bressem et al., 2022). Through these assays, despite the limited available sequencing data, it has been possible to ascribe this viral pathogen

to an unclassified genus within the *Chordopoxvirinae* subfamily, which subsequently includes two described groups: CePV-1 in odontocetes and CePV-2 in mysticetes (Sacristán et al., 2018b). Hence, these characteristic skin blemishes have been distinguished in a notable number of cetacean species, from small cetaceans such as porpoises to larger ones like southern right whales (Baker, 1992; Van Bressem et al., 1993; Raga et al., 1999; Bracht et al., 2006; Fiorito et al., 2015; Yang et al., 2015; Cocumelli et al., 2018). Even though this skin disease does not exclusively affect free-ranging cetaceans, only a few studies have reported its emergence in cetaceans under human care, in contrast to what has been described in wild populations (Flom and Houk, 1979; Ridgway, 1984; Cao et al., 2017; Terio et al., 2018). Nevertheless, the accessibility of marine mammals in housed conditions might provide an opportunity to gain a more detailed view of a wide range of features, which in this regard may contribute to a better understanding of CePV host–pathogen interactions and their possible effects on cetaceans' health. Hence, the aim of the present study was to implement the use of CCS, a non-invasive skin sampling device, as an alternative to skin biopsies by validating their efficacy in detecting CePV in "tattoo-like" lesions in cetaceans in managed facilities. This is intended to corroborate tattoo skin disease (TSD) through evidence-based methods and prove cetaceans in house conditions to serve as an attainable model to improve our knowledge of the health of these marine mammals.

2 Materials and methods

2.1 Study animals

Eighteen bottlenose dolphins (*Tursiops truncatus*), which were housed in two different outdoor pool enclosures in two public display facilities in the Canary Islands, Spain, participated in the present survey. In addition, eight dolphins were held in Rancho Texas Lanzarote Park (Facility 1, FAC1), forming a pod only composed of males. Loro Parque (Facility 2, FAC2) housed the remaining ten animals, where both female and male dolphins formed the group. Dolphins vary in age, establishing three different categories based on their reproductive history (Robeck et al., 2008; Ijsseldijk et al., 2019). Generally, most of the animals were born under human care, specifically second-generation offspring, and they have been kept in almost two different zoos throughout their lives. Both establishments held bottlenose dolphins in five interconnected closed life support pools with a total volume of salt water of more than 7 million liters and a support system of chlorine and ozone. Aside from the temperature of the water, which was regularly monitored to prevent it from exceeding 25°C, dolphins experienced normal fluctuations in environmental conditions, including the day/night cycle and weather temperature.

In FAC1, two study animals (FAC1-N6 and FAC1-N7) had recently been moved from an indoor facility in Germany precisely one month before starting this study. At first, these animals were maintained separately from the main group to comply with appropriate quarantine procedures. During the study, they were gradually allowed to join the social group and subsequently allowed to mix with the other animals, always being controlled by trained animal care staff. Regarding dolphins held in FAC2, dolphin enclosures were freely connected, leaving the gates open, allowing animals to voluntarily perform social mixes within the female and male groups. Sometimes, staff coordinated those mixes to avoid unpredictable breeding or to manage training and medical sessions.

2.2 Sample collection

Skin sample collection was performed at different times throughout the year in 2019. Further, three different visits to FAC1 were conducted in the months of April, October, and December, while one visit was undertaken to FAC2 at the end of October. Unlike animals from this last facility, those housed in FAC1 showed tattoo-like lesions that appeared over different periods of time, which allowed for both macroscopic appraisal and sampling over time. Table 1 provides an overview of general animal information and their individual contributions to the current survey. Skin was sampled from both tattoo-like lesions and apparently healthy skin using CCS, which are sterile plastic swabs with a tip coated with a brush with soft-like texture that rapidly and efficiently dislodge cells in much the same way as described in Segura-Göthlin et al. (2021) (Segura-Göthlin et al., 2021), but with the difference that the present study was accomplished on bottlenose dolphins under professional care in place of stranded cetaceans. Samples were collected by the same person on every visit: the head dolphin trainer or the principal investigator of the present study. Moreover, they were collected individually and on a one-to-one basis in order to prevent cross-contamination. Collecting samples from apparently healthy skin was performed with the aim that they could serve as a control in each sampling procedure.

Sloughed skin was collected by gently brushing the surface of the epidermis of the lesions (Figures 1A, B). With the objective of standardizing the sampling protocol, samples of skin that presumably did not show any lesions macroscopically were intended to be collected at the same location for each individual. Thus, a considerable amount of loose skin was easier to obtain from the dorsal fin of the individuals, in addition to being an area where samples can be collected without the risk of being submerged in water. Consecutively, CCS tips from each sample were introduced into 1.5 ml sterile RNAse- and DNA-se-free microcentrifuge tubes (Thermofisher Scientific, Madrid, Spain), to which 1 ml of RNA*later* Stabilization Solution (Thermofisher Scientific, Madrid, Spain) had previously been added, aiming to preserve both RNA and DNA qualities. Plastic stems were cut to the level of the microcentrifuge tubes' tops, allowing the closure of the vials with the bristled top of the swabs embedded in RNA*later* inside of them. Microcentrifuge tubes were stored at room temperature until their subsequent molecular analysis, which was performed within a working week.

Skin samples from this study were collected in parallel with photo identification of the skin lesions and animals. Firstly, whole body images were taken from each side of the dolphins, aiming to have a correct perspective of the localization as well as the size of the

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TABLE 1 Overview of the study animals and collected skin samples.

NAME	AGE	SEX	BORN	FACILITY	SKIN SAMPLES	LOCATION	ORIGIN	COLLECTION DATE		
								APRIL	OCTOBER	DECEMBER
FAC1-N1	A	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	Melon	Tattoo-like	–	–	✓
FAC1-N2	A	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	Melon	Tattoo-like	–	–	✓
FAC1-N3	A	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	Peduncle	Tattoo-like	–	–	✓
FAC1-N4	A	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	Melon	Tattoo-like	–	–	✓
FAC1-N5	J	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	Dorsal fin	Tattoo-like	–	✓	✓
FAC1-N6	J	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	center ear	Tattoo-like	✓	✓	✓
					A2	Peduncle	Tattoo-like	✓	✓	✓
					A3	Tip	Tattoo-like	–	✓	✓
FAC1-N7	J	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	Blowhole	Tattoo-like	–	✓	✓
					A2	center eye	Tattoo-like	–	✓	✓
FAC1-N8	J	M	Dolphinarium	FAC1	A0	Dorsal fin	Healthy skin	✓	✓	✓
					A1	Dorsal side	Tattoo-like	–	✓	✓
FAC2-N1	A	M	Wild	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N2	A	F	Wild	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N3	A	F	Wild	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N4	A	M	Dolphinarium	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N5	A	F	Dolphinarium	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N6	A	F	Dolphinarium	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N7	A	M	Dolphinarium	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N8	J	F	Dolphinarium	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N9	A	M	Dolphinarium	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–
FAC2-N10	C	M	Dolphinarium	FAC2	A0	Dorsal fin	Healthy skin	–	✓	–

* A, adult; C, calve; F, female; FAC1, facility 1; FAC2, facility 2; J, juvenile; M, male; ✓, sample collected; –, sample not collected.

lesions. To achieve this, dolphins were voluntarily beached or positioned on the surface of the water along one side of the edge of the pool. Furthermore, close-up images allowed for an improved evaluation of the appearance of the tattoo-like lesions.

DNA extraction from sloughed skin samples was carried out through the DNeasy™ Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) with some adaptations as thoroughly explained in Segura-Göthlin et al. (2021) (Segura-Göthlin et al., 2021). Subsequently, the molecular detection of CePV-1 was performed using a real-time polymerase chain reaction (q-PCR) method to amplify a conserved region (150 bp) of the DNA polymerase gene by using the degenerate primer sets designed by Sacristán et al. (2018a) (Odontopox-F: 5'-CARGAAATMAAAAAGAARTTTCCATC-3', and Odontopox-R: 5'-ACGTTCTGTTAARA AYCGTCTTAGTA-3'). Negative (nuclease-free water) and positive controls previously confirmed by our group for both extraction and amplification were included. The PCR products from positive lesions were purified using a commercial kit (Real Clean Spin kit 50 Test-REAL) and then sequenced using Sanger DNA sequencing (Secugen S.L., Madrid, Spain). The amplicon identities were confirmed with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

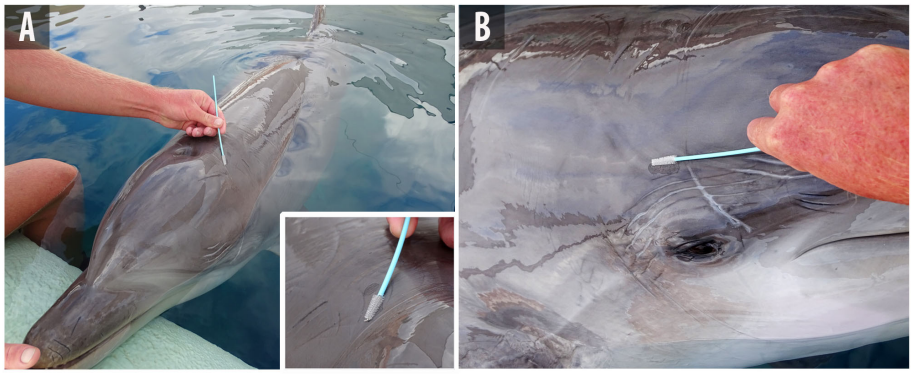

FIGURE 1
Non-invasive skin sampling of tattoo-like lesions in a housed bottlenose dolphin (FAC1-N7) with cytology cell samplers at Facility 1. **(A)** FAC1-N7 is voluntarily rearing its head on the surface of the water by the edge of the pool, allowing a trainer to collect skin samples. Lower inset: close-up detail of the gentle brushing of two near tattoo-like lesions located adjacent to the blowhole. **(B)** While FAC1-N7 is maintaining the same position, the trainer can collect sloughed skin from a second tattoo-like lesion that is located over the right eye.

3 Results

3.1 Study animals and sample collection

In the present survey, eleven samples from tattoo-like skin lesions that were evolving and/or showed different stages that appeared and remitted during three different periods of 2019 were repeatedly collected, obtaining, therefore, a total of 21 samples from those lesions. Furthermore, ten apparently healthy skin samples from animals belonging to FAC2 were collected with eight periodically collected skin samples from the housed dolphins in FAC1. In this way, 34 skin samples were acquired without macroscopical evidence of being affected. This resulted in the collection of 55 skin samples.

3.1.1 First sample collection

A first sample collection was undertaken in April 2019 at FAC1. In this instance, ten skin samples were collected: two from tattoo-like lesions and eight from apparently healthy skin. FAC1-N6, one of the males that came from a facility in Germany one month before starting the present study, showed multiple and variable tattoo-like skin lesions all over the body on the first day it joined the dolphinarium. However, by the time the study started, only two remaining tattoo-like lesions on the left ear (A1) and the right side of the peduncle (A2) were present. Macroscopically, both lesions were oval-shaped, well delimited with a stippled pattern in the center, and associated with rake marks (Figure 2). The first lesion (Figure 2A) was smaller in size (3.5 × 2.2 cm) and slightly clearer than the second one (4.5 × 4.5 cm), which was gray in color with black borders (Figure 2D). Similarly, in the case of FAC1-N7, another male that had also recently arrived at the dolphinarium, all tattoo-like lesions apparently went into remission. Thus, after just one month since these individuals joined the dolphinarium, almost all lesions disappeared. Accordingly, both tattoo-like lesions from FAC1-N6 were collected using CCS. Additionally, eight other samples of apparently healthy skin collected from the dorsal fins of each of the eight animals that formed the pod were also sampled. By this time, FAC1-N6 and FAC1-N7 were independent of the rest of the social group.

3.1.2 Second sample collection

A total of 26 skin samples were collected in October 2019. Eight samples were from tattoo-like lesions and 18 from supposedly healthy skin from both FAC1 and FAC2 individuals. Lesions from FAC1-N6 were systematically sampled. The lesion associated with the left ear doubled in size (5.5 × 3.5 cm), apparently coalescing with another new tattoo-like lesion that seemingly appeared and was superimposed over the initial lesion (Figure 2B). On the other hand, a lesion localized on the peduncle apparently remained in the same aspect (Figure 2E). Rake marks remained perceptible in both lesions. Furthermore, a third tattoo-like lesion appeared on the right dorsal flank of the animal and was collected (1.5 × 1 cm). On this second visit, FAC1-N7 showed multiple tattoo-like lesions on the melon and both flanks. They were different in size, and most were associated with rake marks. Two of those lesions were sampled, which were situated on the right side of the melon just beneath the blowhole and over the right eye. The first was two close-up tattoo-like lesions (1.5 × 1 cm and 2 × 1 cm) (Figure 3A), and the second seemed to show up as three tattoo-like lesions that had coalesced between each other (2.5 × 1 cm) (Figure 3C). During the summer and part of autumn, these two bottlenose dolphins were gradually introduced to the main group during staff-controlled sessions, participating in training and medical sessions as well as the show. Other two animals of the pod (FAC1-N2 and FAC1-N8) showed tattoo-like lesions that were particularly small (1 × 0.5 cm and 0.5 × 0.5 cm, respectively) (Figures 3E, G). Generally, they appeared on the melon of the dolphins and were also associated with rake marks. A total of two skin samples were collected from the lesions of both animals. A fourth individual (FAC1-N5) presented an irregular pale blemish that corresponded to the regression stage of a tattoo-like lesion and was situated dorsal to the right pectoral fin. Additionally, apparently healthy skin samples were collected from all the animals that were integrated into the group. It is important to highlight that the

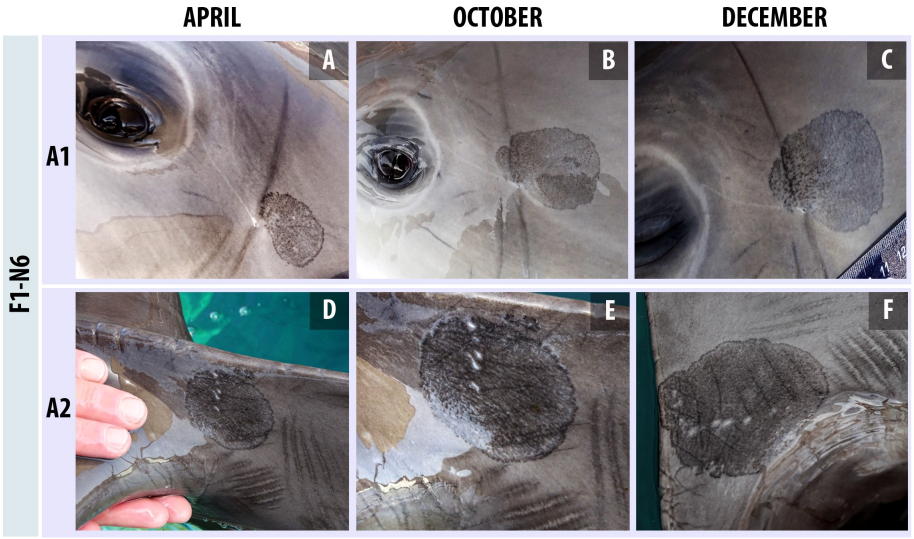

FIGURE 2
Macroscopic development of two tattoo-like lesions between April and December 2019 in a bottlenose dolphin (FAC1-N6) at Facility 1. **(A–C)** Progress of lesion A1: a delimited oval tattoo-like lesion with a stippled pattern in the center that appeared to be associated with the left ear of the dolphin. **(A)** The lesion shows a black border with an evident pattern of dark dots in the center, but with some unaffected clear areas (3.5 × 2.2 cm) in April. **(B)** In October, a newly formed oval tattoo-like lesion appears associated with the initial one, increasing its size (5.5 × 3.5 cm). The border is still evident, but its black color is not that accentuated. A copious stippled pattern affecting the whole interior area of the lesion is observed. **(C)** The lesion has slightly increased in size (6 × 4 cm). The area where the new lesion appeared is slightly raised with marked dark dots in contrast to the rest of the blemish. **(D–F)** Progress of lesion A2: a delimited oval tattoo-like lesion with a stippled pattern in the center, associated with rake marks, and located on the peduncle of the dolphin. **(D)** The lesion is well-delimited with a thick black border, and an inner pinpoint pattern that is darker in the center than the closer areas of the margins (4.5 × 4.5 cm). **(E)** In October, the lesion apparently did not evolve significantly. **(F)** The lesion shows a slight increase in size with a thinner border and a diffuse stippled pattern (5 × 6.5 cm).

increase in interaction marks in every animal in the pod was notable compared to the beginning of the study.

3.1.3 Third sample collection

In December 2019, the eight skin lesions described previously were consistently collected among the other three new ones, in addition to the other eight samples obtained from skin without macroscopical evidence of being affected, obtaining in this manner a total of 19 skin samples. Concerning tattoo-like lesions from FAC1-N6, both lesions from the ear and peduncle continued to increase. The lesion associated with the left ear reached 6 × 4 cm at the end (Figure 2C), and the one on the peduncle was 5 × 6.5 cm (Figure 2F). In addition, the third tattoo-like lesion that appeared on the dorsal right flank associated with a significant incision apparently maintained the same size but darkened slightly in color. Apparently, lesions collected from the melon of FAC1-N7 remained the same (Figure 3B) in contrast to the others that appeared on the right eye, which, by this time, appeared almost completely coalesced (2.5 × 1 cm) (Figure 3D). It must be noted that we observed the increased presence of rake marks with associated tattoo-like lesions on both flanks (Figure 4A). Lesions from FAC1-N2 and FAC1-N8 also continued to evolve until the day of sampling (Figures 3F, H). The lesion on FAC1-N2 located on the melon was darker in color and slightly enlarged (1.8 × 1.2 cm) with a well-marked dark border (Figure 3F). The associated rake marks were no longer perceptible. A small, new tattoo-like lesion was also observed on the tip of the same individual. The lesion from FAC1-N8 appeared to coalesce with another tattoo-like lesion with the same aspect as the initial one (1.2 × 0.8 cm) (Figure 4A). Furthermore, the amount of interaction marks in the melon and flank areas of this individual was remarkable, which, in turn, presented multiple small, associated pinpoint lesions (Figures 4B, C). By this time, the apparently regressive tattoo-like lesion that FAC1-N5 previously showed was no longer observable. Despite the foregoing, skin samples from this area were collected. The other three remaining individuals in the group also showed tattoo-like lesions, which were systematically sampled. FAC1-N3 and FAC1-N4 presented multiple pinpoint lesions associated with rake marks all over the body (Figure 4D). FAC1-N1 showed small tattoo-like lesions on the melon. Moreover, up to this point, all animals had formed a unique group and shared all the pools.

3.2 Molecular findings

From the 55 skin samples collected in the present survey, a total of 31 resulted in positive identification of CePV-1 (56.36%). Specifically, this pathogen was molecularly isolated in 20 of the 21 total samples collected from the eleven tattoo lesions in the present study (95.23%) and in eleven of the 34 total apparently healthy skin samples (32.35%). Table 2 specifies the samples, individual animals, and time at which CePV-1 was detected. On the first visit to FAC1, the two tattoo-like lesions that were observable on FAC1-N6 were positive for CePV-1. Subsequently, in October, there were five more tattoo lesions from four different individuals (FAC1-N2, FAC1-N5, FAC1-N7, and FAC1-N8) and the resurging of a new lesion from

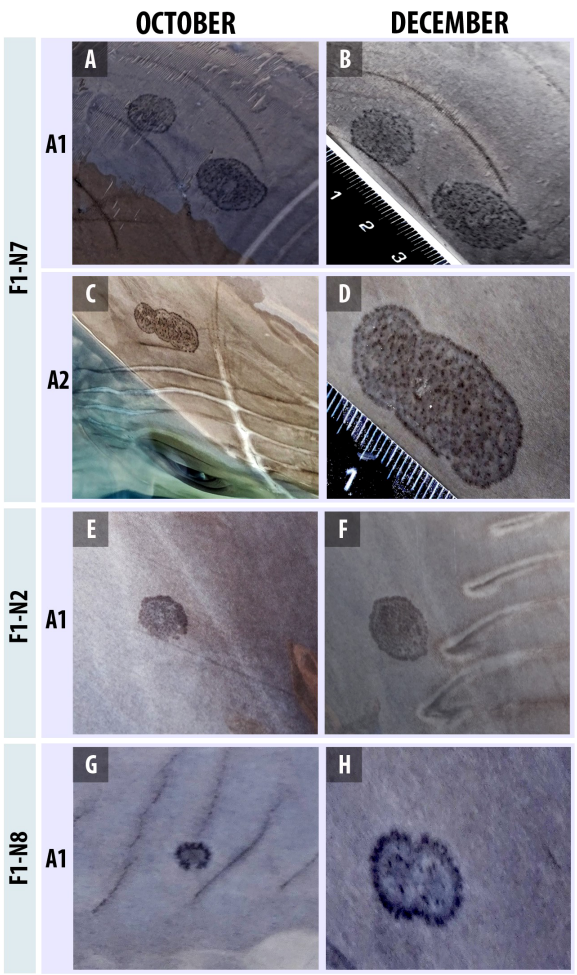

FIGURE 3
Macroscopical development of four tattoo-like lesions between October and December 2019 in three bottlenose dolphins (FAC1-N7, FAC1-N2, and FAC1-N8) at Facility 1. **(A, B)** Progression of lesion A1; tattoo-like lesion on the melon of dolphin FAC1-N2. **(A)** An oval tattoo-like lesion with a marked dark pinpoint pattern on the dorsal part of it, which could correspond to the beginning of the emergence of the margins of the lesion (1 × 0.5 cm). **(B)** In December, the lesion appeared well-delimited and slightly bigger in size (1.8 × 1.2 cm). **(C, D)** Progression of lesion A1; two nearby tattoo-like lesions located close to the blowhole and associated with rake marks on dolphin FAC1-N7. **(C)** Lesions showed slender black margins with a pronounced inner pinpoint pattern and were almost the same size (left lesion: 1.5 × 1 cm; right lesion: 2 × 1 cm). **(D)** In December, both lesions showed hardly any changes, neither in aspect nor size. **(E, F)** Progression of lesion A2: three coalesced tattoo-like lesions associated with rake marks and located next to the right eye on dolphin FAC1-N7. **(E)** In October, three oval tattoo-like lesions that apparently are coalescing between each other can be clearly distinguished, still appreciating the black outer margins of each lesion (2.5 × 1 cm). **(F)** In December, those lesions had almost completely merged with each other, with the margins that visually separated them (2.5 × 1 cm). **(G, H)** Progression of lesion A1; tattoo-like lesion on the melon of dolphin FAC1-N8. **(G)** Small tattoo-like lesion with marked margins that seemingly does not fully limit the lesion. It shows a dark center without a stippled pattern, and it is associated with rake marks (0.5 × 0.5 cm). **(H)** In December, the lesion appears to have coalesced with another with the same appearance and an increase in size (1.2 × 0.8 cm). Rake marks were no longer perceptible.

FAC1-N6, from which CePV-1 was isolated. Thus, considering that detection of CePV-1 was continuedly achieved in the two previous lesions from FAC1-N6, CePV-1 was isolated in a total of eight samples by this time. Those lesions continued to be positive on the third visit. Additionally, at that time, samples from three newly tattoo-like lesions that appeared on three more dolphins from the pod (FAC1-N1, FAC1-N3, and FAC1-N4) were collected and were also positive for CePV-1. In this way, a total of ten samples tested positive for CePV-1 on this last visit. To summarize, 20 samples collected from eleven tattoo-like lesions at different timepoints were positive for CePV-1. On the other hand, CePV-1 was also isolated in eleven of the 34 skin samples collected from which tattoo lesions were not macroscopically identified. Those samples were collected in every visit performed at FAC1 and FAC2. They were sampled from the same body part of each dolphin, specifically the dorsal fin, aiming to standardize the sampling method and avoid areas where lesions frequently appeared. In this manner, despite CePV-1 not being detected on either of the skin samples collected at the first visit, its isolation was achieved in October 2019 from six individuals: two bottlenose dolphins from FAC1 and four from FAC2. Furthermore, on the last visit carried out on FAC1, another five samples obtained from presumed healthy skin were positive for CePV-1. Consequently, it appears that, over time, isolation of CePV-1 from more skin samples is achieved, whether from tattoo-like lesions or apparently healthy skin from individuals with FAC1.

A wide range of cycle threshold values (Ct) are observed. Among samples collected from tattoo-like lesions, the one from which higher viral loads were obtained was the lesion that appeared on the melon of FAC1-N2 on the second visit performed, with a Ct value of 15.35. In contrast, FAC1-N5 presented with lesions with a lower viral load and a Ct value of 35.25 in December. It is important to note that this lesion appeared in an apparently regressive state, showing a slightly paler, almost imperceptible color than the surrounding skin. Focusing on the dolphins whose skin lesions persisted and were sampled more than once, it is notable that they generally showed lower Ct values during the first sampling compared to the following collections, which could indicate that viral loads decreased over time. Hence, while lesions A1 from FAC1-N2 and FAC1-N8 showed Ct values of 15.35 and 18.20 in October, both values slightly increased to 17.94 and 23.13, respectively. Moreover, lesions A1 and A2 collected from FAC1-N6 remained with similar Ct values during the two first collections but were slightly raised in December. Additionally, a third lesion that appeared on the tip of the same animal in October and was repeatedly sampled in December presented a noted decrease in viral loads, changing from showing 16.10 to 30.55 Ct values, respectively. Accordingly, an evident correlation between these molecular results and the gross appearance of the latter lesion can be established, as it macroscopically evolved from a tattoo-like pattern into an irregular pale regressive lesion, which could explain the lower viral load from the last collection. The same association could be made with the regressed lesion of FAC1-N5, which turned out to show Ct values of 30.19 to 35.25 when it was barely distinguishable. Importantly, FAC1-N7 was the only animal that showed an increased viral load in one of its lesions (A2) at the end of the study, which might suggest possible reactivation of the lesion. Notwithstanding the observed slight increases in Ct values in tattoo lesions over time, lesions generally maintained a high viral load.

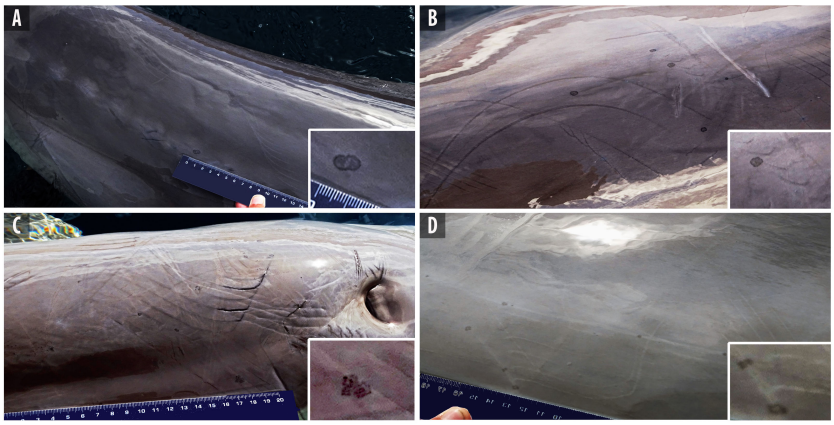

FIGURE 4
Rake marks with associated tattoo-like and pinpoint lesions in three bottlenose dolphins at Facility 1. **(A)** Several short and nearly healed discontinuous interaction marks were followed one by another along the right lateral side of FAC1-N3. Lower inset: zoomed-in image of two coalesced tattoo-like lesions associated with barely noticeable interaction marks. **(B)** Several apparently superficial rake marks are randomly arranged, tracing different trajectories over the right lateral flank of FAC1-N7. Lower inset: note some small, tattoo-like lesions disposed close to rake marks. **(C)** Numerous rake marks showing different healing stages on the caudal area of the blowhole of FAC1-N8. A few almost imperceptible, small, pinpoint, and tattoo-like lesions are associated with the wounds. Lower inset: zoomed-in image of three coalesced tattoo-like lesions associated with rake marks. **(D)** Nearly healed, clear rake marks distributed in different directions on the right flank of FAC1-N8. Lower inset: with this interaction, small tattoo-like lesions are associated.

Regarding samples collected from areas of skin where lesions were macroscopically not distinguishable, CePV-1 was detected in almost one-third of the samples (32.35%). All samples from which the pathogen was detected showed low viral loads, with Ct values ranging from 34.24 to 39.33. However, there was one sample that showed a high viral load with a Ct value of 24.89 and was collected from FAC1-N2. Further interpretation of molecular results and their association with the macroscopic appearance of the lesions is considered in the *Discussion* section.

A CePV-1 DNA polymerase product of 98 bp from the 31 positive skin samples of the present survey was obtained. Accordingly, the same sequence was acquired from the eleven TSD-positive bottlenose dolphins of both zoological parks, whether they showed clinical evidence of the disease or not.

4 Discussion

So far, cetacean skin has been considered an essential matrix for understanding and acquiring an approximate approach to the overall health of these marine mammals (Mouton and Botha, 2012; Aubail et al., 2013; Barlow et al., 2019; Van Cise et al., 2020). Among the range of information that it can provide in relation to health, skin diseases constitute one of the most documented concerns, having their emergence related to environmental stressors such as anthropogenic threats, the exposure to persistent pollutants, and climate change, among others (Wilson et al., 1999; Van Bressem et al., 2009a; Bressem et al., 2015; Koch et al., 2018). However, little has been reported regarding skin diseases in cetaceans living in managed facilities (Thurman et al., 1983; Leamaster and Ostrowski, 1988; Ueda et al., 2013; Duignan et al., 2020). Much of the available information is related to free-ranging cetaceans. In view of the above, together with the fact that skin diseases might be both considerably visible and feasible to study in housed conditions in contrast to wild environments, appraising skin diseases in marine mammals under human care could potentially have great value not only in acquiring a better comprehension of their epidemiology and host-pathogen dynamics but also in assessing the overall health of cetaceans (Clegg et al., 2015).

Skin biopsies and sloughed skin collected with scalpels have been the sampling methods of choice with the aim of molecular diagnosis of specific pathogens in skin lesions in cetaceans, whether in the wild or under human care (Flom and Houk, 1979; Palmer et al., 1991; Esperón et al., 2012). Nevertheless, the employment of those techniques has been questioned because of their invasiveness (Harlin et al., 1999; Bearzi, 2000; Parsons et al., 2003; Kiszka et al., 2010; Noren and Mocklin, 2012; Schilling et al., 2022). This is largely due to ethical advances in the research field, which have advocated the refinement of sampling methods to limit the presumed discomfort that they may induce in the animals and safeguard their well-being, among other approaches. Thus, the present survey is one of the few studies that tries to address the importance of monitoring skin diseases in cetaceans under human care by managing to scientifically corroborate the presence of CePV-1 in tattoo-like lesions without compromising the well-being of individuals through the employment of a non-invasive sampling device.

Through CCS, sufficient desquamating epidermis was obtained by rubbing the surface of the skin several times without compromising it. This is in contrast to other skin sampling procedures used on cetaceans under human care that require deep sampling to maximize material recovery, thereby damaging the skin. As has previously been reported (Raga et al., 1999; Geraci and Lounsbury, 2005; Bracht et al., 2006; Van Bressem et al., 2009a; Powell et al., 2018), most tattoo-like lesions were located in the dorsal areas of the animal, mainly on the melon and lateral flanks.

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TABLE 2 Molecular results from the 18 bottlenose dolphins of the present study.

NAME	SKIN SAMPLES	LOCATION	ORIGIN	MOLECULAR RESULTS		
				CePV CT		
				APRIL	OCTOBER	DECEMBER
FAC1-N1	A0	Dorsal fin	Healthy skin	–	38.8	–
	A1	Melon	Tattoo-like	NA	NA	–
FAC1-N2	A0	Dorsal fin	Healthy skin	–	–	30.89
	A1	Melon	Tattoo-like	NA	15.35	17.94
FAC1-N3	A0	Dorsal fin	Healthy skin	–	–	39.33
	A1	Peduncle	Tattoo-like	NA	NA	19.99
FAC1-N4	A0	Dorsal fin	Healthy skin	–	–	–
	A1	Melon	Tattoo-like	NA	NA	25.05
FAC1-N5	A0	Dorsal fin	Healthy skin	–	–	–
	A1	Dorsal fin	Tattoo-like	NA	30.19	35.25
FAC1-N6	A0	Dorsal fin	Healthy skin	–	–	34.96
	A1	Left ear	Tattoo-like	19.16	19.81	24.76
	A2	Peduncle	Tattoo-like	17.26	18.56	21.22
	A3	Tip	Tattoo-like	NA	16.10	30.55
FAC1-N7	A0	Dorsal fin	Healthy skin	–	34.24	34.54
	A1	Blowhole	Tattoo-like	NA	18.18	18.53
	A2	Left eye	Tattoo-like	NA	21.15	18.84
FAC1-N8	A0	Dorsal fin	Healthy skin	–	–	35.57
	A1	Dorsal side	Tattoo-like	NA	18.20	23.13
FAC2-N1	A0	Dorsal fin	Healthy skin	NA	–	NA
FAC2-N2	A0	Dorsal fin	Healthy skin	NA	–	NA
FAC2-N3	A0	Dorsal fin	Healthy skin	NA	–	NA
FAC2-N4	A0	Dorsal fin	Healthy skin	NA	–	NA
FAC2-N5	A0	Dorsal fin	Healthy skin	NA	35.29	NA
FAC2-N6	A0	Dorsal fin	Healthy skin	NA	38.93	NA
FAC2-N7	A0	Dorsal fin	Healthy skin	NA	–	NA
FAC2-N8	A0	Dorsal fin	Healthy skin	NA	38.91	NA
FAC2-N9	A0	Dorsal fin	Healthy skin	NA	–	NA
FAC2-N10	A0	Dorsal fin	Healthy skin	NA	39.14	NA

* CePV, cetacean poxvirus; HV, herpesvirus; CT, cycle threshold; NA, not applicable; –, negative.

In this manner, whether the animals were voluntarily beached or remained at the exterior of the water at any place of the pool, each skin sample was collected without directly touching the water. This likely improved the adherence of sloughed skin to the bristles. Moreover, collection was neither time-consuming nor laborious. This is due, in part, to the high cellular turnover rate of cetacean skin, which facilitates the collection of sloughed skin (Flom and Houk, 1979; Geraci et al., 1979). Additionally, trainers at both participating facilities completed the skin sampling procedure without the need for dolphins to have undergone previous operant conditioning training (Lauderdale et al., 2021b). Thus, gently rubbing their skin with the bristles of the CCS was rather similar to scratching, which is a positive stimulus commonly used in training sessions at dolphinariums as a supportive reward (Clegg et al., 2019). Likewise, through the employment of CCS, further isolation of CePV-1 in the present survey was attained. Hence, this device could be considered a reference for an innovative sampling method to collect enough epidermal material for the molecular isolation of pathogens without creating discomfort or perturbing animal wellbeing.

Almost all bottlenose dolphins from FAC1, at different times, showed similar tattoo lesions with their characteristic round to oval shape and stippled pattern at the center. Generally, lesions were small and appeared solitary, mostly on the melon or lateral flanks of the animals. Macroscopically, almost all the sampled lesions did not generally undergo any substantial change other than a part of them turning slightly darker with widening borders and barely enlarging as previously described (Sacristán et al., 2018b), and certain ones coalescing between each other. However, there was one individual (FAC1-N5) that showed a tattoo lesion that, at the time of collection, was hardly perceptible and had apparently evolved to a healed stage, according to the denotation given in prior descriptions (Van Bressem et al., 2009b; Sacristán et al., 2018b), to finally disappear on the third visit. With this, it is understood that the macroscopic progression of the lesions of a housed social group continuously sharing the same environment might be divergent and independent of the rest of the pod, observing different stages of the lesions between animals. Thus, while some lesions remained persistent during the same period, others had disappeared. This suggests that what has previously been reported about tattoo lesions can progress in a distinctive manner over time (Geraci et al., 1979; Van Bressem et al., 2008). Furthermore, the might also be applied from an individual standpoint because, besides showing persistent tattoo lesions, animals also presented other poxvirus-like lesions that appeared and rapidly remitted in a short period of time, impeding their collection on time.

Between the months of April and December 2019, molecular isolation of CePV-1 was attained from ten tattoo-like lesions repeatedly collected over time from seven bottlenose dolphins held in FAC1. Thus, molecular corroboration of TSD was achieved in seven of the eight animals. Correspondingly, there was one individual (FAC1-N1), for whom, despite the identification of tattoo lesions at the end of the study, isolation of CePV-1 could not be achieved. However, notwithstanding this negative result, it is highly likely that this dolphin presented with TSD as all the members of the social group contracted the infection over time. This is likely since an inappropriate sampling collection was carried out, resulting in the collection of insufficient sloughed skin. Another possible explanation may be that an incorrect genomic DNA procedure on this skin sample was unintentionally conducted. Either way, excluding the latter, all tattoo lesions sampled in the present study resulted in a positive diagnosis of CePV-1.

One of the most notable findings of the present study was the molecular isolation of CePV-1 from apparently healthy skin. Thus, skin that does not show macroscopic evidence of lesions compatible with poxvirus from the dorsal fin of the 18 bottlenose dolphins in the current study was collected along with the sampling of tattoo-like lesions, aiming to serve as a control for further DNA extraction. Unexpectedly, this resulted in the detection of CePV-1 in eleven apparently healthy skin samples collected from five individuals from FAC1 and four dolphins housed in FAC2. To our knowledge, this is the first report in which the isolation of CePV-1 from apparently healthy skin in cetaceans is achieved. Further, in a prior study carried out by Melero and co-workers (Melero et al., 2014), performed on another marine mammal, specifically a Pacific walrus (*Odobenus rosmarus divergens*) held under human care, the authors detected poxvirus in the skin and in other organs such as the pre-scapular and tracheobronchial lymph nodes and tonsils, without the animal showing gross lesions. The amplicons obtained had a striking similarity to CePV-1. Inevitably, the molecular detection of CePV-1 in the absence of clinical evidence raises several questions. A possible explanation for these results might be related to the high sensitivity of q-PCRs, which can sometimes highlight increased probabilities of contamination and possible subsequent false-positive data (Opota et al., 2015). Contamination can be due to the circulating cell-free DNA brought from the environment, which raises another question of whether the detection of CePV-1 was due to the presence of viral particles in the water interfering with the sample rather than its isolation in apparently healthy skin. However, in order to detect the virus in this medium, specific protocols for pathogen concentration are normally required, as has been proven in multiple studies focused on the isolation of infectious agents that persist and are transmitted by aquatic means (Albinana-Gimenez et al., 2009; Girones et al., 2010). In addition, even though PCR is a very sensitive detection technique, in zoological conditions, pools are subjected to continuous disinfection treatments, which might significantly reduce the concentration of the virus (Girones et al., 2010; Lanrewaju et al., 2022). However, CePV-1 was also detected in apparently healthy skin samples from bottlenose dolphins from FAC2, a completely different scenario where tattoo-like lesions had not been reported in any of the dolphins. To date, thanks to the medical history carried out both by veterinarians and with the help of trainers, none of the dolphins on this establishment have ever shown clinical signs of the disease, and no molecular diagnostics of CePV have been performed. Accordingly, these results suggest that the latter individuals may have been developing the skin disease in a subclinical manner. Thus, CePV-1 could be present or even proliferating, resulting in the absence of clinical signs of the disease, which may be due to a low infective dose of the viral agent, and/or good animal health and/or the immunological status of the infected individuals, thus impeding disease progression. Correspondingly, Ct values from these apparently healthy skin samples showed remarkably low viral loads, ranging from 35.29 to 39.14, which could be one of the reasons why the skin disease did not eventually develop. With this last hypothesis, it could be deduced that low infective concentrations of the pathogen could have been present in areas of skin where there was no clinical evidence of poxvirus-like lesions in the bottlenose dolphins from FAC1, and that a route of entry through damaged skin may have been necessary for the development of the lesion. In this context, it is observed that Ct values obtained from these supposedly healthy skin samples indicated significant low viral loads, with values ranging from 30.89 to 38.8, which could support the hypothesis above stated. However, caution must be exercised when making these assumptions, as the present study, to our knowledge, is the first reported to date that has attempted to perform molecular analyses to detect poxvirus in apparently healthy skin in cetaceans.

Interestingly, the same sequence was derived from both tattoo lesions and skin samples without clinical evidence of the disease in dolphins from both FAC1 and FAC2 facilities. Besides, this amplicon showed a high homology of 99.10% and a query cover

of 100% to the published sequence obtained from bottlenose dolphins in Brazil by Sacristán and co-workers (GenBank accession no. KU726612). A plausible interpretation of the fact that bottlenose dolphins kept under managed care showed a strain of CePV-1 with such remarkable similarities to another obtained from wild populations might reside in the likelihood that this pathogen has persisted through generations among housed cetaceans worldwide since the introduction of original wild-caught individuals around the 90s (Van Waerebeek et al., 2006), with the capability to be latent and cause subclinical infections in individuals without giving rise to the skin disease until reactivation by certain stimuli. Although there is no scientific evidence that corroborates TSD in the originally introduced and captured wild bottlenose dolphins, most of which came from Caribbean populations (Fisher and Reeves, 2007; Brownell and Reeves, 2008), prior reports have isolated CePV-1 sequences with high similarities to the one in question from social pods throughout the North and South Atlantic Seas (Sacristán et al., 2018a; Luciani et al., 2022), strengthening the possibility of the persistence of the virus along this geographical area. Furthermore, apparently this strain does not exclusively infect bottlenose dolphins, as quite homologous sequences have been detected in an Atlantic spotted dolphin and in a striped dolphin stranded along the Canary and Mediterranean coasts (Sacristán et al., 2018a; Segura-Göthlin et al., 2021). In this manner, the fact that it might have been transferred among different free-ranging cetacean species could indicate a high incidence of infection in wild populations. Moreover, supporting the latter hypothesis, a recent study from Rodrigues and co-workers (Rodrigues et al., 2020) achieved the isolation of a CePV-1 sequence from classic tattoo-like lesions from a managed Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) kept in an oceanarium in Hong Kong, which also showed maximum likelihood with the above-mentioned strain (Bracht et al., 2006). The latter not only supports what is already known about the high distribution of this viral skin disease and the wide range of cetacean species that may be affected in the wild, but also raises the presumption that the same could also be happening in cetaceans held in zoos and aquariums around the world.

Little has so far been reported about the incidence of TSD in cetaceans under human care, which could represent a breakthrough in our understanding of the disease and the health of animal populations in captivity and in the wild. Up until now, some studies have suggested that environmental factors could influence the emergence of tattoo-like lesions in housed individuals. Thus, it has been found that water temperature changes at facilities that are in places where they experience notable fluctuations in the weather contribute to the emergence of tattoo-like lesions. In this way, elevated water temperatures favor the remission of the lesions, while reduced temperatures presumably encourage their presence (Gulland et al., 2018; St Leger et al., 2018). Comparing these observations with our current findings, it is assumed that the present survey was carried out in two facilities located on the Canary Islands, where subtropical temperatures are mild and stable throughout the year within the range of 18–24°C without noticeable interference with the water conditions (Bechtel, 2016). Certainly, bottlenose dolphins from FAC1 were gradually contracting the skin disease between the months of April and December, being that almost all animals were TSD-positive at the end of the year, coinciding with the winter season. However, it is very likely that this may be due to the progressive introduction of two CePV-1-positive bottlenose dolphins (FAC1-N6 and FAC1-N7) to the rest of the group, which could have led to the spread of the disease rather than mere variations in water temperature. Nevertheless, it is important to note the almost complete disappearance of the tattoo lesions in these two individuals during their first weeks of arrival at the enclosure, which could have been linked to environmental differences between the facility these dolphins came from and the one they were introduced to. Furthermore, the introduction of two new members of the pod caused a sustained reestablishment of the social hierarchy and, in turn, a gradual increase in interaction marks, which is normal behavior among this species (Scott et al., 2005; Clegg et al., 2015; Clegg et al., 2019). This survey reinforces what has previously been described in studies of cetaceans under managed care, noting that a great part of the emerging tattoo lesions in the present study were associated with rake marks (Van Bressem et al., 2017; St Leger et al., 2018). This suggests these discontinuities in the skin as a potential route of entry for the pathogen (Van Bressem et al., 2008; Mouton and Botha, 2012; Savini et al., 2017). In this manner, as interaction marks between animals increased, a higher prevalence of lesions was observed, as well as TSD-positive individuals.

Correspondingly, the current survey has served to probe whether CCS is a practical device in that it allows skin samples to be taken promptly and non-invasively without causing harm or affecting the well-being of the animals during the collection, being assertive in isolating CePV-1 from sloughed skin. Moreover, it is adaptable for trainers and caretakers due to the ease of performing the sampling and the unnecessary need to restrain or even capture the animals during clinical/health assessments, avoiding the stress that these procedures entail. Taken together, this CCS is a conceivable and innovative tool to enhance veterinary and husbandry practices to assess the health of captive cetaceans, which is an important target for zoos and aquariums. Furthermore, through this device, it has been possible to confirm with scientific corroboration what has previously been reported in relation to the epidemiology of this skin disease. Thus, tattoo lesions macroscopically evolve and persist on cetacean skin in an independent and indefinite manner and seem to recurrently disappear and appear. This supports hypotheses regarding the persistent nature of CePV infections due to a possible latent phase for a considerable period, where the virus is quiescent until reactivation is triggered by environmental stimuli or health conditions, as it could occur with the increase of intraspecific interactions due to imbalances in the social environment or fluctuations in water temperatures. However, even though this research constitutes one of the few prospective studies held on CePV in cetaceans under human care, it must be noted that additional longitudinal studies of this skin disease should be carried out to gain better scientific knowledge concerning host–pathogen interaction dynamics. Hence, in this article, we illustrate the significance of developing research in cetaceans under human care, which could be of significant value in taking steps towards improving and understanding animal health both in housed

conditions and in the wild, and to progress on the conservation of these marine mammals.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GenBank, OQ199540.

Ethics statement

Ethical review and approval was not required for the animal study because Skin sample collection from the housed cetaceans was carried out by the first author of the present manuscript as a qualified researcher and veterinarian, and the trainers authorized by Loro Parque and Lanzarote Rancho Texas Park zoos, within their routine medical program, as well as their use for the present scientific study, in compliance with the requirements established on the articles 3, 4 and 5 of the "Ley 31/2003, de 27 de octubre, de conservación de la fauna silvestre en los parques zoológicos" (BOE-A-2003-19800) and the Council Directive 1999/22/EC of 29 March 1999 relating to the keeping of wild animals in zoos (EUR-Lex -31999L0022). In addition, the use of this skin samples for the isolation of cetacean poxvirus was for the current investigation was expressly authorized by the Directors of Loro Parque and Lanzarote Rancho Texas Park zoos.

Author contributions

Conceptualization, ES, AF, and JA. Methodology and formal analysis, SS-G, ES, AF, MA, IF-J, and AC-R. Writing-original draft preparation, SS-G and ES. Supervision, ES, AF, MA, and JA. Funding acquisition, AF, ES, MA, and JA. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Gabriele Rossi,
Murdoch University, Australia

REVIEWED BY
Pádraig Joseph Duignan,
Marine Mammal Center, United States
Carlos Sacristán Yagüe,
Centro de Investigación en Sanidad Animal (CISA), Spain

*CORRESPONDENCE
Marisa Ana Andrada Borzollino
✉ marisaana.andrada@ulpgc.es

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Viral skin diseases in odontocete cetaceans: gross, histopathological, and molecular characterization of selected pathogens

Simone Segura-Göthlin[1], Antonio Fernández[1], Manuel Arbelo[1], Marisa Ana Andrada Borzollino[1]*, Idaira Felipe-Jiménez[1], Ana Colom-Rivero[1], Carla Fiorito[2] and Eva Sierra[1]

[1]Veterinary Histology and Pathology, Atlantic Center for Cetacean Research, University Institute of Animal Health and Food Safety (IUSA), Veterinary School, University of Las Palmas de Gran Canaria (ULPGC), Las Palmas, Spain, [2]Centro para el Estudio de Sistemas Marinos, Consejo Nacional de Investigaciones Científicas y Técnicas (CESIMAR-CONICET), Puerto Madryn, Chubut, Argentina

Fifty-five skin lesions from 31 stranded cetaceans along the Canary coasts (2011–2021) were submitted to macroscopic, histological, and molecular analyses to confirm infection by cetacean poxvirus, herpesvirus and cetacean morbillivirus. They were macroscopically categorized into eight categories with respective subcategories according to their color, shape, size, and consistency. Cetacean poxvirus was detected in 54.54% of the skin lesions through real-time and conventional PCRs based on the DNA polymerase gene. Additionally, herpesvirus and morbillivirus were currently detected from 43.63 and 1.82% of the cutaneous lesions, respectively. Coinfection of poxvirus and herpesvirus was detected in nine of them (16.36%), which makes the present study the first to report coinfection by both pathogens in skin lesions in cetaceans. A plausible approach to histopathological characterization of poxvirus-and herpesvirus-positive skin lesions was established. Hyperkeratosis, acanthosis, ballooning degeneration, and intracytoplasmic inclusion bodies in vacuolized keratinocytes through the stratum spinosum were common findings in poxvirus skin lesions. Alphaherpesvirus was associated with a prominent acanthotic epidermis, moderate necrosis, multifocal dyskeratosis, and irregular keratinocytes with both cellular and nuclei pleomorphism. The common histopathological findings of both pathogens were observed in coinfection lesions. However, those associated with herpesvirus were considerably more remarkable. Relationships between molecular and microscopic findings were observed for the lesions that showed tattoo-like and tortuous patterns. Further multidisciplinary diagnostic studies of infected skin lesions are needed to understand the epidemiology of these emerging infectious diseases.

KEYWORDS

cetacean poxvirus, coinfection, herpesvirus, histopathology, molecular diagnosis, morbillivirus, skin lesions

1. Introduction

Cetaceans have long life spans, resident and transient strategies, and high trophic levels that make them promising as sentinels that reflect large-scale aquatic ecosystem health (1–4). The long-term investigations of these marine mammals in the past two decades have facilitated the documentation in wild populations of several diseases, including those caused by emerging or re-emerging pathogens (5, 6). Researchers consider cetacean epidermal conditions as useful for evaluating species health and environmental status (2, 7, 8). Skin diseases are among the most well-documented diseases that affect cetacean species globally (9). Apart from their high visibility, they are of particular scientific interest for several reasons: (1) some microorganisms affecting the skin are considered opportunistic, as they invade and infect pre-existing wounds, leading to the progression of distinctive skin lesions or systemic infections (10–12); (2) the prevalence and persistence of skin diseases in these marine mammals relates to host immunologic dysfunction resulting from chronic exposure to anthropogenic factors, distress, and other infectious diseases (13–15); and (3) they usually involve a broad spectrum of pathogens (16).

Several cutaneous lesions have been associated with viruses in free-ranging cetaceans, including the cetacean poxvirus (CePV) (17). CePV causes the most widely reported and globally prevalent skin disease and is typically diagnosed through visual assessment (18–20). CePV has a distinctive clinical presentation characterized by flat or slightly raised hyperpigmented oval patches that may be solitary or coalescing and give the appearance of "ring-like" lesions (21). However, CePV can also present with an irregular stippled pattern, commonly referred to as a "tattoo" lesion, which prompted the categorization of this disease as tattoo skin disease (TSD) (22). CePV may reflect generalized immune suppression in cetacean populations, making it a potential indicator of cetacean health (4, 23, 24). Herpesvirus (HV) infections in cetaceans are more commonly associated with systemic infections (25–27) and encephalitis (28–30) related to the *Alphaherpesvirinae* subfamily (alphaherpesvirus) (31). Nevertheless, gammaherpesviruses have also been detected in genital and skin lesions with different manifestations in cetaceans (32), ranging from flat oval lesions and proliferative wounds to raised verrucous nodules and plaque-like lesions, respectively (11). Regarding skin disorders, HV in cetaceans has been associated with different types of dermatitis, such as proliferative, fibrinosuppurative, and necrotizing dermatitis (33–35). Viral skin diseases have been less frequently associated with papillomaviruses that cause proliferative nodules (18), calicivirus-inducing vesicular disease (36–38), and morbilliviruses skin lesions along with severe respiratory, nervous, and immune impairments (6, 39, 40). Morbilliviruses are among the most significant emerging pathogens of cetaceans globally and cause lethal disease outbreaks with extensive geographic distributions among very large host populations of cetaceans (39, 41).

Nevertheless, skin lesion assessments are challenging for free-ranging cetaceans because of their limited accessibility in the wild and the costly and time-consuming investments required (42–44). Hence, most studies have relied on long-term photographic surveys to evaluate the progression and course of skin diseases (13, 45, 46). Observational or photographic surveys are, however, considered suboptimal, and ancillary diagnostic tests are required to determine the causative agent of a skin disorder even when the macroscopic manifestation is assumed to be characteristic or pathognomonic of a specific etiology (47–49). Accordingly, most studies strictly associate CePV with typical tattoo-like lesions, disregarding other possible skin manifestations that can be triggered by this virus. This leads to limited genomic information with which to correctly designate this pathogen (20, 50, 51). Likewise, restricting the detection of this pathogen from tattoo-like lesions reduces the probability of identifying co-infections from macroscopically different lesions. On this premise, the detection of pathogens from skin lesions would enable genomic characterization and phylogenetic analysis and facilitate a better understanding of the epidemiology of these pathogens.

The aim of the present study is a complete molecular screening of poxvirus and other viruses, such as herpesvirus and cetacean morbillivirus, in various skin disorders from stranded cetaceans in the Canary Islands. Additionally, macroscopic, histological, and molecular examinations, in conjunction with phylogenetic analysis, were performed to provide insights about these emerging infectious skin diseases.

2. Materials and methods

This was a retrospective study, and skin samples were selected from cetaceans with good to moderate states of preservation, and/or the collection of both formalin-fixed and fresh unfixed portions from each skin sample. Accordingly, skin samples ($n = 55$) from 31 cetaceans stranded on the coast of the Canary Archipelago, Spain, from March 2011 to May 2021 were analyzed. Six different species of cetaceans were included in the present study, including striped dolphins (*Stenella coeruleoalba*; $N = 10$), Atlantic spotted dolphins (*Stenella frontalis*; $N = 9$), common dolphins (*Delphinus delphis*; $N = 4$), common bottlenose dolphins (*Tursiops truncatus*; $N = 3$), short-finned pilot whales (*Globicephala macrorhynchus*; $N = 3$), and Risso's dolphins (*Grampus griseus*; $N = 2$). All study samples were subjected to standardized necropsies, and the decomposition code, conservation methods, and other data (including sex and age) for each animal were obtained according to standard guidelines (52–55). Five decomposition codes were established: code 1 (extremely fresh) to code 5 (mummified or skeletal) (55). Most animals had a good state of preservation (code 2), while four animals were euthanized (56, 57) because of a poor prognosis and provided extremely fresh carcasses (code 1). Nevertheless, for management reasons, it was not always possible to perform necropsies of individuals preserved at room temperature, and some animals were kept frozen to avoid further decomposition prior to necropsy. Based on the total body length and histological gonadal development, the age categories were classified as follows: neonate, calf, juvenile, and adult (58, 59). Additionally, stranding and epidemiological information (type, location, and date) were also systematically recorded and have been summarized in Supplementary Table S1. Notably, four animals in the present study have been previously published; poxvirus was detected in three of these animals (cases 2, 27, and 30; CETS 601, 1,151, and 1,173, respectively) and herpesvirus was detected in another (case 25; CET 1103). During necropsies, formalin-fixed and fresh unfixed samples of representative tissues, including skin samples, were collected for histopathologic and molecular analysis, respectively (60). Fixed tissues were submitted in 10% neutral buffered formalin solution, processed, embedded in paraffin blocks, and sectioned into 5 µm slices before

staining with hematoxylin and eosin (HE). Fresh unfixed samples were stored at −80°C before being selectively submitted for virological testing and mycological and bacteriological analyses. For the latter, slices were cultured on Sabouraud agar and morphologic colony identification was performed along with routine culture and surface plating on Columbia blood agar; the API system was used for preliminary identification of isolates (54, 60). The epibionts, ectoparasites, and endoparasites were preserved in 70% alcohol for parasitological analysis. The identification relied on macroscopic, submacroscopic, and histologic features (60, 61).

2.1. Macroscopic analysis of skin lesions

All skin lesions were described, measured, and photographed. Their locations on the body were recorded along with their macroscopic appearance, color, shape, and consistency.

2.2. Molecular analysis of skin lesions

For each study animal, 0.5 g of fresh-frozen skin sample was added to 500 µl 1X cell lysis buffer (Cell Signaling Technology, United States) and 4.5 ml of diethylpyrocarbonate (DEPC)-treated water (Ambion, Invitrogen) for two consecutive rounds of mechanical homogenization at 3549 ×*g* with a 30-s rest interval in a Precellys 24 tissue homogenizer (Bertin Technologies SAS, France). The homogenized samples were centrifuged at 2163 ×*g* for 15 min at 4°C in a high-speed refrigerated benchtop centrifuge (Megafuge series, Thermo Fisher Scientific, Waltham, MA, United States). Total DNA/RNA extraction from each 300 µl macerated sample was performed using a QuickGene Mini 80 nucleic acid isolation machine (QuickGene, Kurabo, Japan) according to the manufacturer's instructions, with a slight modification: RNA carrier (Applied Biosystems, Thermo Fisher Scientific) was added during the lysis step as previously described (62).

CePV-1 molecular detection from 55 extracted samples was performed using two different assays. First, semi-quantitative polymerase chain reaction (sqPCR) based on SYBR green was used to amplify a conserved region (150 bp) of the odontocete poxvirus DNA polymerase gene using the degenerate primer sets designed by Sacristán and coworkers (20). To assess specificity, a conventional PCR amplification of the 543-bp fragment from the *Chordopoxvirinae* subfamily (capri-, sui-, cervid-, and ortho-poxvirus) DNA polymerase gene of the qPCR CePV positive samples was also performed using the primer sequences originally designed by Bracht and collaborators (50). PCR products (5 µl per sample) were read on a 2% agarose electrophoresis gel containing GelRed (Biotium, Inc., California, United States).

Panherpesvirus conventional nested PCR was performed for HV detection using the universal HV nested PCR protocol originally developed by VanDevanter and coworkers (63). Additionally, to obtain semi-quantitative data on viral loads of each sample, a nested SYBR Green sqPCR for HV detection was carried out using the same degenerate primers as above to amplify a 200-bp region of the DNA polymerase gene as in conventional PCR (29). A 4-µL aliquot from the DNA extraction was amplified in a mixture containing 10 µl of 2X SsoAdvanced Universal SYBR Green Supermix with a high-fidelity Taq DNA polymerase based on Bio-Rad's patented Sso7d fusion protein technology (Bio-Rad Laboratories, Inc., California, CA, United States), 250 nM of each primer, 1x GC-RICH solution (Roche Diagnostics S.L., Barcelona, Spain), and nuclease-free water to bring the final volume to 20 µL. The reactions were set for 3 min of polymerase activation at 98°C, followed by 45 amplification cycles, each comprising a denaturation step at 95°C for 15 s, an annealing step at 46°C for 30 s, and an elongation step at 72°C for 1 min. The final cycle was composed of an extended elongation at 72°C for 7 min. Thereafter, 5 µL of the amplicons from the second PCR were read by 2% agarose gel electrophoresis to corroborate the sq-PCR results.

Furthermore, total RNA extracted from the 55 skin samples was submitted for molecular detection of the Cetacean Morbillivirus (CeMV) through sq-PCR using primers targeting highly conserved fragments of the phosphoprotein gene (205 bp), as previously described (41). Two negative and positive controls (for extraction and amplification) were included in each protocol.

PCR products were purified using a Real Clean spin kit (REAL, Durviz, S.L., Valencia, Spain) for sequencing (Secugen S.L., Madrid, Spain). Sequencing used 1 µl (5 µM) of each of the following primers: Odontopox-F and Odontopox-R for CePV-1 (20), TGV (internal forward) and IYG (internal reverse) for HV (63), and PAN-F and PAN-R for CeMV (41). Amplicon identities were confirmed with BLAST.[1]

The cycle threshold (Ct) values for the CePV and HV sq-PCRs, which consisted of the target-specific amplification signals, were determined to assess viral loads and the risk of transmission and recovery (64). Late Cts (typically cycles 30–45) are near the limit of detection and are considered marginally positive (65). Ct values are inversely related to viral loads; greater concentrations of viral genetic material require fewer cycles of amplification (66). Nevertheless, caution should be taken when evaluating this factor as poor DNA extraction and/or nucleic acid degradation can affect results. Melting curves were used to confirm the amplification of the dsDNA products.

2.3. Phylogenetic analysis

The sequences of HV and CePV were aligned (excluding primers) with the Clustal W algorithm using MEGA X software (Pennsylvania, PA, United States) (67, 68). A total of 99 and 29 HV and CePV-1 nucleotide sequences, respectively, were recovered from GenBank to construct the phylogenetic trees. Both trees were established from deduced nucleotide sequences using the Maximum Likelihood Method. Accordingly, for HV, the Tamura 2-parameter model with a discrete Gamma distribution was used to model the evolutionary rate differences among sites (5 categories (+G, parameter = 0.7779)). The Tamura 3-parameter model with a Gamma parameter of 0.2836 was used for modeling the CePV tree (67). Bootstrap consensus trees were inferred from 500 replicates. Although branches corresponding to partitions reproduced in <50% of bootstrap replicates are collapsed, only values >70% were considered meaningful.

1 www.ncbi.nlm.nih.gov/blast/Blast.cgi

2.4. Histopathological analysis of skin lesions

Thirty-three of 55 (69.1%) skin lesions were considered for histologic analysis (including lesions that were positive and negative by a molecular test for any of the three pathogens). To accurately relate histopathological changes with the viruses involved, skin lesions that histologically showed coinfection by other etiological agents such as bacteria or protozoa ($n = 6$) were not considered. This also applied to skin lesions associated with traumatic wounds ($n = 1$). Carcasses that were too compromised to submit to freezing preservation ($n = 7$) or that were too advanced in decomposition code ($n = 4$) were not considered because of artifacts unavoidably induced by the freeze–thaw process and tissue autolysis. Moreover, samples from four skin lesions were not available for histopathological analysis.

The frequent histopathological findings associated with viral skin infections were graded as follows: absent (−), minimal (+), mild (++), moderate (+++), and severe (++++) (69). Plausible associations of histological observations with macroscopic appraisals, as well as molecular findings, were investigated.

Immunohistochemistry techniques (IHC) targeting HV and CeMV were also performed on respective positive skin lesions as complementary diagnostic assays. Thus, serial sections (3 µm thickness) were sliced and stained as previously described (29, 70). Appropriate positive and negative immunohistochemical controls were included for both IHCs.

3. Results

3.1. Macroscopic findings of skin lesions

The skin lesions were categorized as shown in Table 1. The most observed pattern was the tattoo-like oval shape lesion (TL-O), followed by black-fringed (BF) and white-fringed (WF) lesions. The remaining categories were rather equally reported, except the pale pattern (P), which was rarest. The lesions were predominantly on the heads and both flanks of cetaceans, though lesions were also found on the fins and the ventral regions. Generally, lesions were of different sizes, and animals rarely had multiple lesions. Twenty skin lesions were associated with discontinuities of the skin (40%), which were mostly rake marks (for a better appreciation see Supplementary Table S2).

3.2. Molecular findings of skin lesions

Of the 55 skin lesions, 46 were positive (83.63%) for one or more of the selected viruses, and nine were negative (16.36%; see Supplementary Figure S1). CePV-1 was exclusively detected in 21 (38.18%) of the skin lesions, HV was present in 15 (27.27%; 13 were positive for alphaherpesvirus and two for gammaherpesvirus), and evidence of CeMV was found in only one (1.82%). CePV-1 and HV coinfection was detected in nine of the 55 skin lesions (16.36%; see Table 2).

Overall, 11 of the 31 cetaceans tested exclusively positive for CePV-1 (35.48%); eight were solely positive for HV (25.80%), and CeMV was detected in only one (1.82%). Both HV and CePV-1 viruses were simultaneously detected in eight animals (25.80%). Among these, CET 1151 presented with four lesions, of which two were coinfected. Three cetaceans tested negative for the selected pathogens (9.67%).

A range of Ct values (12.01–38.41) were observed for lesions testing positive for CePV-1 by sq-PCR. For HV-positive lesions, Ct values also ranged widely (19.27–37.60). Generally, coinfected lesions had high Ct values, which were not too divergent for both pathogens.

All macroscopic skin categories were positive for one or more of the selected pathogens (see Table 2). The highest number of positive lesions (whether CePV-1 and/or HV positive) was for the TL-O ($N = 12$). None of these lesions tested negative, which was also true of TL-S lesions ($N = 5$). Seven lesions categorized as WF and BF tested positive for selected pathogens. The remaining macroscopic categories tested positive at similar rates, apart from category P which had only one lesion (which tested positive). All gross categories had similar numbers of negative lesions (one or two).

CePV-1 was present in every subcategory of tattoo-like lesions, as well as in WF, BF, and R lesions. Aside from TL-C lesions, HV was detected in all the remaining macroscopic categories. CeMV was detected in a BF lesion. CePV-1 and HV coinfection was mostly detected in tattoo-like lesions (TL-O and TL-S; $N = 7$).

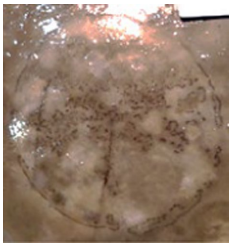
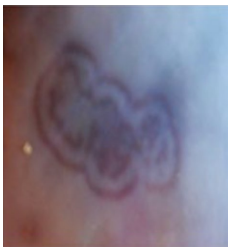
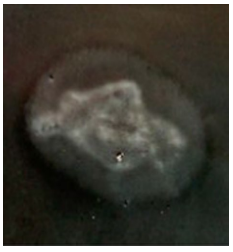
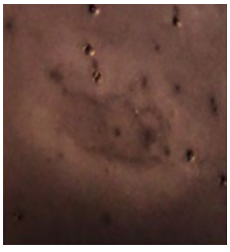
3.3. Phylogenetic findings

In this study, 36 sequences were obtained: 19 CePV-1 and 16 HV sequences based on the polymerase genes, and one CeMV phosphoprotein gene sequence (summarized in Table 2). Nine CePV-1 DNA polymerase products (353–524 bp) and ten other amplicons with shorter lengths (77–99 bp) were obtained. Figure 1 shows the corresponding phylogenetic tree in which only longer sequences and dereplicated sequences were considered. The phylogenetic tree was formed from seven amplicons along with 25 CePV-1 and two CePV-2 GenBank sequences, with the addition of two outgroup sequences (a skunkpox virus and a raccoonpox virus). The sequence obtained from the common dolphin (ON600453) clustered together (bootstrap value (BV) of 98%) with five sequences from common dolphins stranded in the United Kingdom and one Indo-Pacific bottlenose dolphin. Two CePV-1 sequences from a Risso's dolphin (ON600456) and a short-finned pilot whale (ON600457) of our study were clustered together (BV of 96%). The sequence of the common bottlenose dolphin (ON600458) was grouped (BV of 88%) with a sequence detected in another animal of the same species. The sequence of the striped dolphin (ON600454) was in the same cluster (BV of 95%) with four other sequences from striped dolphins from the United Kingdom and Italy and one harbor porpoise stranded in the United Kingdom. Regarding the sequences obtained of the Atlantic spotted dolphin species in our study, one of them (ON600451) did not cluster with any other sequences of the phylogenetic tree, while the other (ON600459) clustered (BV of 95%) with a sequence obtained from a Guiana dolphin stranded in Brazil.

Amplicons ($n = 16$) with 193, 191, 190, 181, and 169 bp were identified from the 24 skin lesions that tested positive for HV (Supplementary Figure S2). Three large clusters (one for *gammaherpesvirus* and two for alphaherpesvirus sequences arising from the same branch supported by a BV of 91%) comprising several of the HV sequences were identified in the phylogenetic tree

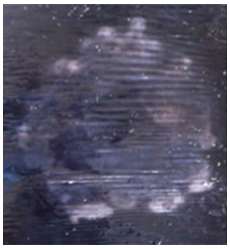
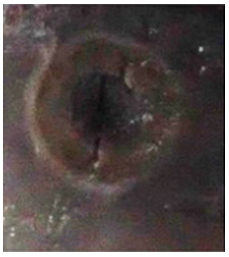
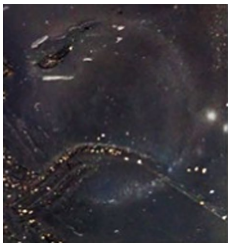
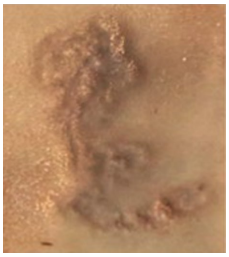
(Figure 2A). Gammaherpesvirus sequences ($n = 2$) were clustered together among other sequences from the same herpesvirus subfamily with a relation of 97% (Figure 2B). Remarkably, both sequences were closely related to a virus detected in a penis lesion of a striped dolphin stranded in the same geographic area (GenBank KM248274). Regarding alphaherpesvirus sequences, one large

TABLE 1 Macroscopical classification of skin lesions from the present study with their corresponding gross findings.

Category		Description	Gross-findings	Incidence	
				Lesions ($n = 55$)	Percentage (%)
1. Tattoo-like	a. Oval-shaped	Round to irregular well-marked lesions with dark margins and stippled pattern in the centre.	 Case 16 (CET 995) *Grampus macrorhynchus* Lesion A1	12	21.81
	b. Coalesced (49)*	Oval-shaped lesions that have coalesced between each other.	 Case 2 (CET 601) *Stenella frontalis* Lesion A1	3	5.45
	c. Serpiginous	Multiple small stippled black lesions very closely located between each other or even coalesced. Their unification and distribution resulted into a serpiginous appearance.	 Case 3 (CET 642) *Stenella frontalis* Lesion A1	5	9.09
2. Black-fringed (19)*		This category refers to those round lighter patches in contrast to the average coloration of the skin, with blurred black margins. Occasionally, they presented a slightly dark pinhole or irregular jagged pattern in the centre.	 Case 21 (CET 1056) *Stenella frontalis* Lesion A1	9	16.36
3. White-fringed (19)*		This category comprised those round black blemishes or normally colored skin with fade whitish margins. In some cases, an irregular pattern can be present in the centre of the lesions.	 Case 26 (CET 1138) *Stenella frontalis* Lesion A1	8	14.54

(Continued)

TABLE 1 (Continued)

Category	Description	Gross-findings	Incidence	
			Lesions (n = 55)	Percentage (%)
4. Pale (19)*	This category refers to pale in color and irregular in shape lesions.	 Case 25 (CET 1103) *Tursiops truncatus* Lesion A2	1	1.81
5. Ulcerative (11)*	Irregular shaped open skin lesions with completely loss of the epidermis.	 Case 20 (CET 1045) *Delphinus delphis* Lesion A4	4	7.27
6. Target-like (16)*	This category presented oval lesions with dark margins and depressed centre that occasionally could be eroded or ulcerated.	 Case 26 (CET 1138) *Stenella frontalis* Lesion A4	3	5.45
7. Ring (11)*	Included in this category were oval flat lesions with uniform divergent colors from black, grey, to white, and even almost imperceptible blemishes that have acquired the color of the normal skin.	 Case 28 (CET 1152) *Stenella frontalis* Lesion A1	6	10.90
8. Tortuous	This category refers to black or white linear lesions setting out tortuous tracts. Additionally, they can show depressed or raised pattern.	 Case 23 (CET 1067); Lesion A3	4	7.27

*N, number of lesions. Asterisks indicate references from which these categories have been previously established.

cluster (BV 73%; Figure 2C) contained seven sequences from our study, with three obtained from the common bottlenose dolphin, three from the Atlantic spotted dolphin, and one from the Risso's dolphin species. All sequences, except one from a common bottlenose dolphin (OM454361), were in well-supported groups with other sequences obtained from animals of the same species, with BVs > 70%. Concerning sequence OM454361, it was clustered with a BV of 97% with sequences detected in several cetacean

TABLE 2 Molecular results from the 55 skin lesions of the 31 animals stranded on Canary coasts between 2011 and 2021 tested on the present study.

Case N.	ID code	Species	Lesion	MC	PCR results			CT values			Sequences		
					CePV-1	HV	CeMV	CePV-1	HV	CeMV	CePV-1	HV	CeMV
					N = 21/55	*N* = 15/55	*N* = 1/55						
1	CET 566	*S. coeruleoalba*	A1	WF	–	–	–	–	–	–	–	–	–
2	CET 601	*S. frontalis*	A1	TL-C	+	–	–	22.89	–	–	ON600451	–	–
3	CET 642	*S. frontalis*	A1	TL-S	+	+	–	**19.24**	**27.19**	–	**ON600452**	**OM456331**	–
4	CET 663	*D. delphis*	A1	TL-O	+	–	–	20.95	–	–	ON600453	–	–
5	CET 705	*S. coeruleoalba*	A1	TL-S	+	–	–	22.09	–	–	ON600454	–	–
6	CET 748	*S. coeruleoalba*	A1	TL-S	+	–	–	32.10	–	–	ON600455	–	–
7	CET 751	*G. griseus*	A1	T-LO	+	–	–	20.06	–	–	ON600456	–	–
8	CET 947	*D. delphis*	A3	TL-O	+	+	–	**17.73**	**35.64**	–	**ON600460**	**OM456332**	–
9	CET 951	*S. coeruleoalba*	A1	TL-O	+	+	–	**34.83**	**24.17**	–	**ON600461**	**OM456333**	–
10	CET 959	*S. coeruleoalba*	A1	U	–	–	–	–	–	–	–	–	–
11	CET 969	*G. macrorhynchus*	A6	TL-O	+	–	–	34.72	–	–	ON600462	–	–
12	CET 983	*S. coeruleoalba*	A3	TL-C	+	–	–	35.13	–	–	ON600463	–	–
13	CET 984	*G. griseus*	A4	TL-O	+	+	–	**36.05**	**36.83**	–	**ON600464**	**OM456334**	–
14	CET 985	*S. coeruleoalba*	A1	TL-S	+	+	–	**37.49**	**34.79**	–	**ON600465**	**OM456335**	–
15	CET 991	*S. coeruleoalba*	A3	R	–	–	–	–	–	–	–	–	–
16	CET 995	*G. macrorhynchus*	A1	TL-O	+	–	–	22.04	38.20	–	ON600457	–	–
17	CET 1020	*T. truncatus*	A1	TL-O	+	+	–	**13.79**	**35.55**	–	**ON600466**	**OM456336**	–
18	CET 1035	*S. coeruleoalba*	A2	BF	–	–	+	–	–	22.32	–	–	**ON314830**
19	CET 1044	*S. frontalis*	A1	R	–	+	–	–	29.21	–	–	OM456337	–
20	CET 1045	*D. delphis*	A4	U	–	+	–	–	37.60	–	–	OM456338	–
21	CET 1056	*S. frontalis*	A1	BF	–	+	–	–	24.75	–	–	OM456339	–
22	CET 1058	*S. frontalis*	A1	BF	+	+	–	**38.41**	**33.86**	–	**ON600467**	**OM456340**	–
23	CET 1067	*S. frontalis*	A3	Ts	–	+	–	36.30	31.70	–	–	ON314829	–
24	CET 1069	*S. coeruleoalba*	A1	R	+	–	–	37.21	–	–	ON600468	–	–
25	CET 1103	*T. truncatus*	A2	P	–	+	–	–	35.31	–	–	OM456341	–
26	CET 1138		A1	WF	–	+	–	–	19.27	–	–	OM456342	–
			A2	WF	–	+	–	–	23.90	–	–	OM456342	–
		S. frontalis	A3	T	–	+	–	–	35.80	–	–	OM456342	–

(Continued)

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TABLE 2 (Continued)

Case N.	ID code	Species	Lesion	MC	PCR results			CT values			Sequences		
					CePV-1	HV	CeMV	CePV-1	HV	CeMV	CePV-1	HV	CeMV
					N = 21/55	*N* = 15/55	*N* = 1/55						
			A4	T	–	–	–	–	–	–	–	–	–
			A5	U	–	+	–	–	21.22	–	–	OM456342	–
27	CET 1151	*T. truncatus*	A1	TL-S	**+**	**+**	**–**	**23.65**	**32.60**	**–**	**ON600458**	**OM456343**	**–**
			A3	T	–	+	–	–	–	–	–	OM456344	–
			A4	U	–	+	–	–	29.87	–	–	OM456343	–
			A6	R	**+**	**+**	**–**	**31.80**	**28.52**	**–**	**ON600458**	**OM456344**	**–**
28	CET 1152		A1	Ts	–	+	–	–	36.71	–	–	OM456345	–
			A2	WF	–	+	–	–	21.37	–	–	OM456345	–
		S. frontalis	A3	Ts	–	–		–	–	–	–	–	–
			A4	R	–	+	–	–	37.34	–	–	OM456345	–
			A5	BF	–	+	–	–	34.68	–	–	OM456345	–
29	CET 1153	*D. delphis*	A1	BF	–	–	–	–	–	–	–	–	–
			A2	Ts	–	–	–	–	–	–	–	–	–
			A3	BF	–	–	–	–	–	–	–	–	–
30	CET 1173	*S. frontalis*	A1	TL-O	+	–	–	15.65	–	–	ON600459	–	–
			A2	TL-O	+	–	–	18.08	–	–	ON600459	–	–
			A3	TL-O	+	–	–	16.42	–	–	ON600459	–	–
			A4	BF	+	–	–	33.63	–	–	ON600459	–	–
			A5	BF	+	–	–	25.02	–	–	ON600459	–	–
			A6	WF	+	–	–	33.44	–	–	ON600459	–	–
			A7	WF	+	–	–	31.79	–	–	ON600459	–	–
			A8	R	+	–	–	35.37	–	–	ON600459	–	–
			A9	WF	+	–	–	28.49	–	–	ON600459	–	–
			A10	WF	+	–	–	12.01	–	–	ON600459	–	–
31	CET 1181		A1	TL-O	+	–	–	13.11	–	–	ON600469	–	–
		G. macrorhynchus	A2	TL-C	–	–	–	–	–	–	–	–	–
			A3	BF	+	–	–	27.43	–	–	ON600469	–	–

*CT, cycle threshold; CePV-1, cetacean poxvirus; CeMV, cetacean morbillivirus; HV, herpesvirus; MC, macroscopic classification; A1–A10 = skin lesion samples 1–10; BF, black-fringed; R, ring; T, target-like; Ts, tortuous; TL-C, tattoo-like, coalesced; TL-S, tattoo-like, serpiginous; TL-O, tattoo-like, oval-shaped; P, pale; U, ulcerative; WF, white-fringed; –, negative. CePV-1 and HV coinfected samples are indicated in boldface.

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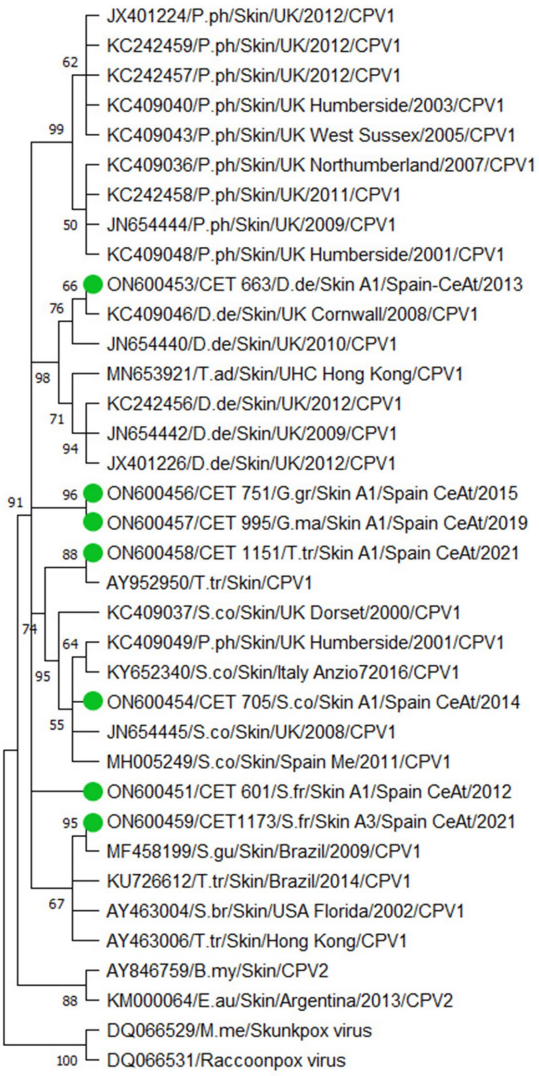

FIGURE 1
Phylogenetic analysis based on 29 nucleotide sequences from the polymerase gene of cetacean poxvirus. Seven sequences obtained from this study are denoted in colored green circles. The accession number, the identification number, the host, the geographic stranding, and the date of collection were used to identify the nucleotide sequences. B.my (*Balaena mysticetus*); D.de (*Delphinus delphis*); E.au (*Eubalaena australis*); G.gr (*Grampus griseus*); G.ma (*Globicephala macrorhynchus*); M.me (*Mephitis mephitis*); P.ph (*Phocoena phocoena*); S.br (*Steno bredanensis*); S.co (*Stenella coeruleoalba*); S.fr (*Stenella frontalis*); T.ad (*Tursiops aduncus*); T.tr (Tursiops truncates) CeAt (Central Atlantic Ocean); Me (Mediterranean Sea). To construct the tree, we designed the Neighbor-Join and BioNJ algorithms along with the Tamura 3-parameter model and Gamma distribution to model the evolutionary rate differences among sites [five categories (+G, parameter = 0.5213)]. The Bootstrap method was performed to resample 500 replicates and evaluate the reliability of the tree.

species that shared some characteristics, including necrosis and the presence of intranuclear inclusion bodies (INIB) in the affected organs. The other large cluster within the *Alphaherpesvirinae* subfamily was supported by a BV of 76% (Figure 2D) and contained six sequences: three were from the Atlantic spotted dolphin. The sequence from the common dolphin was grouped (BV of 75%) with sequences detected in animals of the same species stranded along the coasts of Portugal and Spain. Finally, a sequence detected in a common dolphin in our study (OM454338) was in a separate branch (BV of 97%) in which there are no other sequences detected in the skin. This sequence clustered (BV of 73%) with sequences detected in common dolphins stranded in Portugal and the Canary Islands, an Atlantic spotted dolphin stranded in the Canary Islands, and common bottlenose dolphins stranded in the United States and Germany (Figure 2A). Supplementary Table S3 reveals more concisely the percent identity of each study sequence with the closest GenBank match.

Lastly, sequencing of the P gene fragment of the product obtained from CET 1035 (167 bp) revealed a relation of 100% with DMV detected in the lung of a fin whale (*Balaenoptera physalus*) stranded in Denmark in 2016 (GenBank MH430939), in a Risso's dolphin stranded in the Canary Islands in 2015 (GenBank KY886370) and in a bottlenose dolphin stranded in the United States in 2013 (GenBank KU720622). Additionally, this similarity was observed for sequences derived from the lung, brain, pulmonary and mesenteric lymph nodes, spleen, kidney, and liver samples from striped dolphins stranded in Galicia and Portugal waters.

3.4. Histopathological and immunochemical findings

Thirty-eight of the 55 skin samples were considered adequate for histopathological examination (69.1%; Supplementary Table S4). Based on the analysis of the most prevalent microscopic findings and etiologies (Table 3), acanthosis (68.16%) and ballooning degeneration (54.53%) were considered the predominant histopathological changes in skin lesions positive for CePV-1. Vacuolized epidermal cells were multifocally concentrated in apical areas of this layer or created linear columns (Figure 3A), which rarely expanded laterally to create multifocal cones (Figure 3B). Where ballooning degeneration was observed, simultaneous moderate multifocal hyperkeratosis was typically observed (59.09%; Figure 3C), which in turn was associated with mild focal hyperpigmentation (31.81%). More rarely (27.27%), small, round, irregular, and pale eosinophilic intracytoplasmic inclusion bodies (ICIBs) were observed in vacuolized keratinocytes (Figures 3D,E).

Diffuse hyperkeratosis (40%) with acanthotic epithelium (39.99%) was predominantly found in alphaherpesvirus-positive lesions (Figure 4A). In other cases, the distinctive loss of the stratum corneum and part of the stratum spinosum was observed (Figure 4B). Cellular and nuclear pleomorphisms (Figure 4C), as well as multifocal basophilic syncytial keratinocytes, were observed in the apical areas of the stratum spinosum (Figure 4D). In some lesions (33.33%), the stratum spinosum randomly showed mild, multifocal, well-delimited, oval, necrotic areas concentrated with degenerated keratinocytes and neutrophils (Figure 4E). Severe neutrophilic inflammatory cell infiltration in blood vessels was a common finding (40%), while INIBs were difficult to distinguish in all alphaherpesvirus-positive lesions (6.66%).

Regarding the ICH results, immunostaining for HV was not observed in any of the HV-positive (by PCR) skin lesions even though immunostaining was successful for the positive control. Nevertheless, evidence of INIBs was more definite for CET 1103 after immunolabeling than after HE staining (Figure 4F).

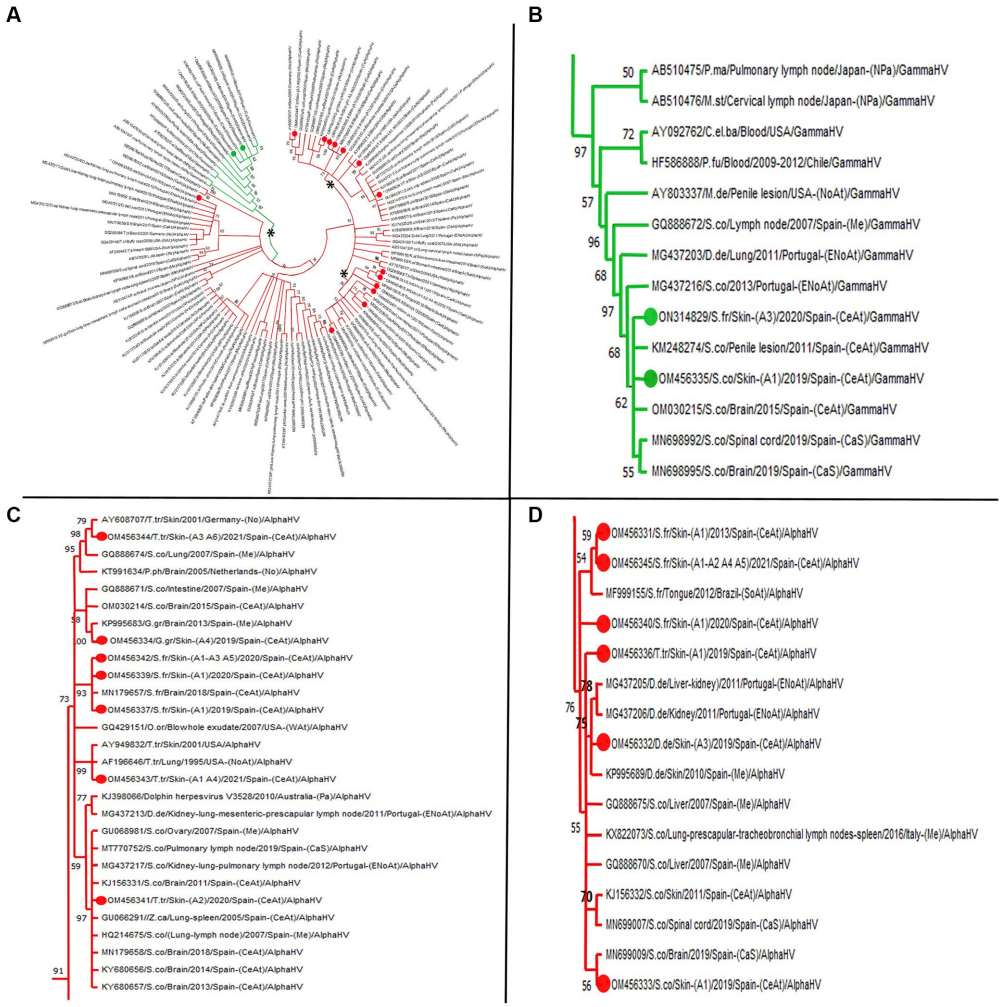

FIGURE 2
Maximum likelihood phylogenetic tree. **(A)** Molecular phylogenetic analysis based on 91 nucleotide sequences from the polymerase gene of cetacean herpesvirus. 16 sequences obtained from this study are denoted in green (gammaherpesvirus) and red (alphaherpesvirus) colored circles. The accession number, the identification number, the host, the geographic stranding, and the date of collection were used to identify the nucleotide sequence. Asterisks remarks representative clusters. **(B)** Clade with 14 GenBank available cetacean gammaherpesvirus sequences among which two were obtained in the present study. **(C,D)** Clades with different bootstrap values grouping most representative alphaherpesvirus sequences obtained. **(C)** Remark sequence with GenBank acc.no. OM456341 obtained from case 25 (skin lesion A2) which shows a 97% similarity with sequences obtained from other tissues rather than skin. **(D)** Note the big clade with bootstrap value of 76, grouping sequences in several subclades according to species. C.el.ba (*Cervus elaphus barbarous*); D.de (*Delphinus delphis*): G.gr (*Grampus griseus*); M.de (*Mesoplodon densirostris*); M.st (*Mesoplodon stejnegeri*); P.fu (*Pseudalopex fulvipes*); S.co (*Stenella coeruleoalba*); S.fr (*Stenella frontalis*); T.tr (*Tursiops truncates*); Z.ca (*Ziphius cavirostris*); P.ma (*Physeter catodon*); P.ph (*Phocaena phocaena*); NoAt (North Atlantic Ocean); ENoAt (Northeast Atlantic Ocean); WAt (West Atlantic Ocean); CeAt (Central Atlantic Ocean); SoAt (South Atlantic Ocean); Me (Mediterranean Sea); CaS (Cantabrian Sea); Pa (Pacific Ocean); NPa (North Pacific Ocean); No (North Sea); ArO (Arctic Ocean). To construct the tree, we designed the Neighbor-Join and BioNJ algorithms along with the Tamura 3-parameter model and Gamma distribution to model the evolutionary rate differences among sites (five categories (+G, parameter = 0.5319)). The Bootstrap method was performed to resample 1,000 replicates and evaluate the reliability of the tree.

In coinfected lesions, a combination of the above-described histologic changes from both CePV-1 and HV pathogens were observed. Diffuse acanthosis was a common finding (66.66%) along with multifocal ballooning degeneration (66.66%) with associated hyperkeratosis (55.55%). Almost all coinfected lesions presented ICIBs in which the typical umbrella-like arrangement or "melanin-cap" was noticeably absent. Conversely, INIBs were only noticed in CET 951, where both ICIBs and INIBs were apparent with obvious multifocal syncytial organizations (Figure 5A). Irregular ICIBs (Figure 5B) and multifocal apoptotic-like keratinocytes were observed through the intermediate layer at a mild to moderate degree (13.34%; Figure 5C). Combined mild to moderate lymphocytic and neutrophilic inflammatory cell infiltration and congestion were observed in several lesions (55.55%).

The CeMV-positive lesion presented with mild acanthosis with a disorganized histologic architecture for which some rete ridges were laterally fused and almost parallel to the stratum spinosum (Figure 6A). Furthermore, this lesion also tested positive by IHC, with a few random keratinocytes lightly immunolabeled for canine distemper virus (CDV; Figure 6B).

Among pathogen-negative lesions, those from CET 1153 showed moderate diffuse acanthosis. Inflammatory cell infiltration (ICI) was multifocally observed in the apical areas of the dermal papillae. The

TABLE 3 Percentages and number of lesions presenting each histopathological finding grouped by etiologies.

Skin associated lesions		CePV-1 (*n* = 22)		HV (*n* = 15)		CeMV (*n* = 1)		Coinfection (*n* = 9)	
		Lesions (*n*)	Percentage (%)	Lesions (*n*)	Percentage (%)	Lesions (*n*)	Percentage (%)	Lesions (*n*)	Percentage (%)
Hyperkeratosis	Minimal	4	18.18	2	13.33	1	100	0	0
	Mild	4	18.18	3	20	0	0	3	33.33
	Moderate	5	22.72	1	6.67	0	0	2	22.22
	Severe	0	0	0	0	0	0	0	0
		13	**59.09**	**6**	**40**	**1**	**100**	**5**	**55.55**
Acanthosis	Minimal	7	31.81	1	6.66	0	0	0	0
	Mild	7	31.81	3	20	1	100	5	55.55
	Moderate	1	4.54	2	13.33	0	0	1	11.11
	Severe	0	0	0	0	0	0	0	0
		15	**68.16**	**6**	**39.99**	**1**	**100**	**6**	**66.66**
Ballooning degeneration	Minimal	6	27.27	0	0	0	0	1	11.11
	Mild	2	9.09	0	0	0	0	3	33.33
	Moderate	3	13.63	0	0	0	0	2	22.22
	Severe	1	4.54	0	0	0	0	0	0
		12	**54.53**	**0**	**0**	**0**	**0**	**6**	**66.66**
Spongiosis	Minimal	2	9.09	0	0	0	0	1	11.11
	Mild	1	4.54	0	0	0	0	0	0
	Moderate	0	0	0	0	0	0	0	0
	Severe	4	18.18	0	0	0	0	0	0
		7	**31.81**	**0**	**0**	**0**	**0**	**1**	**11.11**
Necrosis	Minimal	0	0	3	20	0	0	2	22.22
	Mild	0	0	1	6.66	0	0	0	0
	Moderate	0	0	1	6.66	0	0	1	11.11
	Severe	0	0	0	0	0	0	0	0
		0	**0**	**5**	**33.32**	**0**	**0**	**3**	**33.33**
Satellitosis		**0**	**0**	**1**	**6.67**	**0**	**0**	**1**	**6.67**
Hyperpigmentation	Minimal	4	18.18	1	6.66	0	0	0	0
	Mild	3	13.63	0	0	0	0	0	0
	Moderate	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0
		7	**31.81**	**1**	**6.66**	**0**	**0**	**0**	**0**
Hypopigmentation	Minimal	0	0	1	6.66	0	0	0	0
	Mild	0	0	2	13.33	0	0	0	0
	Moderate	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0
		0	**0**	**3**	**20**	**0**	**0**	**0**	**0**
Fused rete ridges	Minimal	2	9.09	2	13.33	0	0	2	22.22
	Mild	0	0	0	0	0	0	0	0
	Moderate	0	0	1	6.66	1	1	0	0
	Severe	0	0	1	6.66	0	0	0	0
		2	**9.09**	**4**	**26.65**	**1**	**100**	**2**	**22.22**
ICIBs		**6**	**27.27**	**0**	**0**	**0**	**0**	**6**	**66.66**

(Continued)

TABLE 3 (Continued)

Skin associated lesions		CePV-1 (*n* = 22)		HV (*n* = 15)		CeMV (*n* = 1)		Coinfection (*n* = 9)	
		Lesions (*n*)	Percentage (%)	Lesions (*n*)	Percentage (%)	Lesions (*n*)	Percentage (%)	Lesions (*n*)	Percentage (%)
INIBs		**0**	**0**	**1**	**6.66**	**0**	**0**	**1**	**6.67**
Inflammatory cell infiltration	Minimal	8	36.36	3	20	1	100	2	22.22
	Mild	4	18.18	0	0	0	0	3	33.33
	Moderate	1	4.54	0	0	0	0	0	0
	Severe	0	0	3	20	0	0	0	0
		13	**59.09**	**6**	**40**	**1**	**100**	**5**	**55.55**
Congestion	Minimal	4	18.18	2	13.33	0	0	1	6.67
	Mild	2	9.09	2	13.33	0	0	2	22.22
	Moderate	1	4.54	0	0	0	0	2	22.22
	Severe	0	0	1	6.66	0	0	0	0
		7	**31.81**	**5**	**33.32**	**0**	**0**	**5**	**51.11**
Dyskeratosis/ apoptosis	Minimal	4	18.18	1	6.66	0	0	0	0
	Mild	1	4.54	0	0	0	0	1	6.67
	Moderate	0	0	0	0	0	0	1	6.67
	Severe	0	0	0	0	0	0	0	0
		5	**22.72**	**1**	**6.66**	**0**	**0**	**2**	**13.34**
Pearl corns	Minimal	0	0	2	13.33	0	0	2	22.22
	Mild	0	0	1	6.66	0	0	0	0
	Moderate	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0
		0	**0**	**3**	**20**	**0**	**0**	**2**	**22.22**

*CePV-1, cetacean poxvirus; CeMV, cetacean morbillivirus; HV, herpesvirus. Bold indicates the percentages, and underlined numbers represents the highest values.

remaining negative lesions did not show any remarkable histological changes.

The tattoo-like and BF lesions were the most prevalent by microscopy (Table 4), but these histologic changes were mild to moderate. Preliminarily, 75% of the TL-O lesions showed mild acanthosis, followed by mild to moderate hyperkeratosis, ballooning degeneration, and ICIBs (66.66%). All the TL-S lesions presented mild to moderate ballooning degeneration and congestion. A significant proportion of the latter cases (80%) were associated with mild to moderate hyperkeratosis and acanthosis. These two subcategories of tattoo lesions were also among the few in which ICIBs were observed (60%). Of the BF skin lesions, 88.88% showed mild acanthosis, followed by mild to moderate hyperkeratosis (66.66%). A repeated pattern exclusively present in the Ts lesions was observed with well-delimited multifocal areas of degenerative keratinocytes and neutrophils that sometimes merged to the outer layer, leading to mild to moderate disruptions of the stratum corneum. Consequently, 75% of the lesions presented moderate necrosis. Neither ulcered nor target-like lesions are represented in Table 4, as they did not apply to the histologic analysis and/or their histological changes were not evaluable.

4. Discussion

Because of their limited accessibility, most pro-active health studies in free-ranging cetaceans exclusively assess their skin conditions using only visual appraisals for diagnosis (23, 71), which results in a high risk for misinterpretation of skin disease pathogens. Therefore, stranded cetaceans are critical study subjects that provide unlimited access and the opportunity to fully comprehend skin diseases and their impact on the health of marine mammals. Hence, this study represents the first multidisciplinary study involving macroscopic, histological, and molecular analyses of a significant number of viral skin lesions in several species of stranded cetaceans. Molecular identification of CePV in poxvirus-like skin lesions has been performed in several species (20, 51, 72). However, to the authors' knowledge, the present study is the first to identify this virus in pilot whales. HV infections have been identified in several cetacean species and tissue samples (20, 73, 74). However, HV DNA has not been reported in skin lesions of Risso's dolphins, which makes the present study the foremost publication on HV related to skin lesions in this species.

Viral skin lesions in these marine mammals are generally considered potential health indicators (14, 75). Most studies have focused on recognizing TSD lesions because of their wide global distribution and characteristic and distinguishable presentations; the molecular identification of CePV has been associated with these lesions (76, 77). However, to the best of our knowledge, no studies have surveyed viral pathogens other than CePV nor their co-occurrence in CePV-positive cetacean skin lesions. Most studies of CePV coinfection have implicated tissues other than the skin; Melero and co-workers (78) detected both poxvirus and HV in the tonsil of a

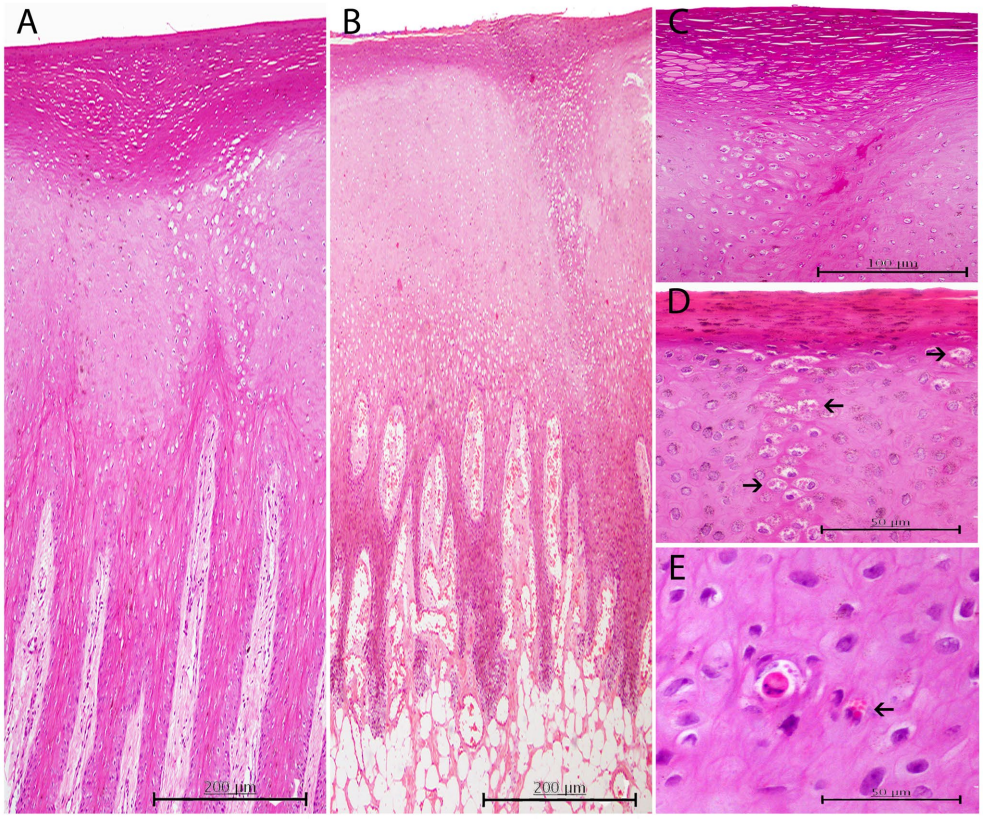

FIGURE 3
Histopathological findings in CePV-1 positive skin lesions from five cases. **(A)** Lesion A1 from case 7. Focal marked hyperkeratosis showing two focal columns of ballooning degeneration affecting apical areas of rete ridges and the epidermal transitional zone between both stratums corneum and spinosum. H and E, ×10. **(B)** Lesion A1 from case 16. Focal zone of moderate ballooning degeneration affecting both stratum corneum and spinosum. Marked hyperkeratosis just above the line of vacuolated keratinocytes is observed. Marked multifocal congestion in the dermal papillae. H and E, ×10. **(C)** Lesion A6 from case 11. Marked focal hyperkeratosis. Beneath this affected area, a moderate focal ballooning degeneration in the stratum spinosum is appreciated. H and E, ×20. **(D)** Lesion A1 from case 30. ICIBs detected in a column-like group of vacuolized keratinocytes (arrows). Right above, mild hyperkeratosis with associated slightly hyperpigmented keratinocytes. HE, ×40. **(E)** Lesion A1 from case 31. Acidophilic apoptotic keratinocyte with small amphophilic ICIBs. Multiple irregular sized ICIBs in a vacuolated keratinocyte (arrow). H and E, ×40.

Pacific walrus (*Odobenus rosmarus divergens*). To our knowledge, this investigation is the first to corroborate HV and CePV coinfection in marine mammals; previous studies of concomitant skin lesion infections by both agents have been conducted in other species such as hares (*Lepus*), while leporipoxvirus and leporid gammaherpesvirus-5 co-infections were recently reported (79). In cattle, an outbreak of lumpy skin disease virus and bovine herpesvirus-4 occurred in Egypt where cows showed generalized deep skin nodules among other clinical signs (80). Reports exist of commercial chicken flocks showing wart-like lesions consistent with fowl poxvirus and severe respiratory manifestations from infectious laryngotracheitis virus (81). HV and CeMV coinfection has been detected in multiple organs of a few cetaceans (28, 82, 83), as well as CeMV and *Brucella* sp. in central nervous system (29, 84). Nevertheless, this is the first report revealing a considerable prevalence of poxvirus (35.48%) and herpesvirus (25.80%) skin diseases in stranded cetaceans in the Canary Archipelago, in addition to providing the first molecular description of CePV and HV coinfection in cetacean skin lesions (25.80%).

As reported in prior studies, the lesions were mostly observed on visible body parts, especially on dorsal areas, with the head being the most affected (50, 85). Of the eight macroscopic categorizations of 55 skin lesions, the tattoo-like pattern was the most predominant, especially the TL-O form. Usually, this pattern is identified as an early manifestation of TSD (22, 23). The molecular results of the study indicate that all lesions with this presentation are positive for CePV-1, and the majority have high viral loads. However, three oval tattoo-like lesions presented with low Ct values, possibly because a non-representative sample of the lesion was processed for genomic extraction, or because of genomic degradation of the sample. Alternatively, the CePV-1 viral loads may have been affected by HV, which was detected in two of those three tattoo-like lesions. As previously reported (22, 77), the dominant histological findings of tattoo-like lesions were mild to moderate ballooning degeneration associated with hyperkeratosis and acanthosis. Additionally, other acute histopathological processes were moderate vascular congestion with the migration of lymphocytes. Of the three tattoo-like subcategories, the TL-O form showed moderate acute histopathological changes. Furthermore, in correlating Ct values with the latter microscopic findings, this macroscopic category showed early CePV-1 amplifications, which could indicate that these lesions may be the initial manifestations of TSD. Finally, ICIBs were observed in all cases of TL-O, suggesting viral activity.

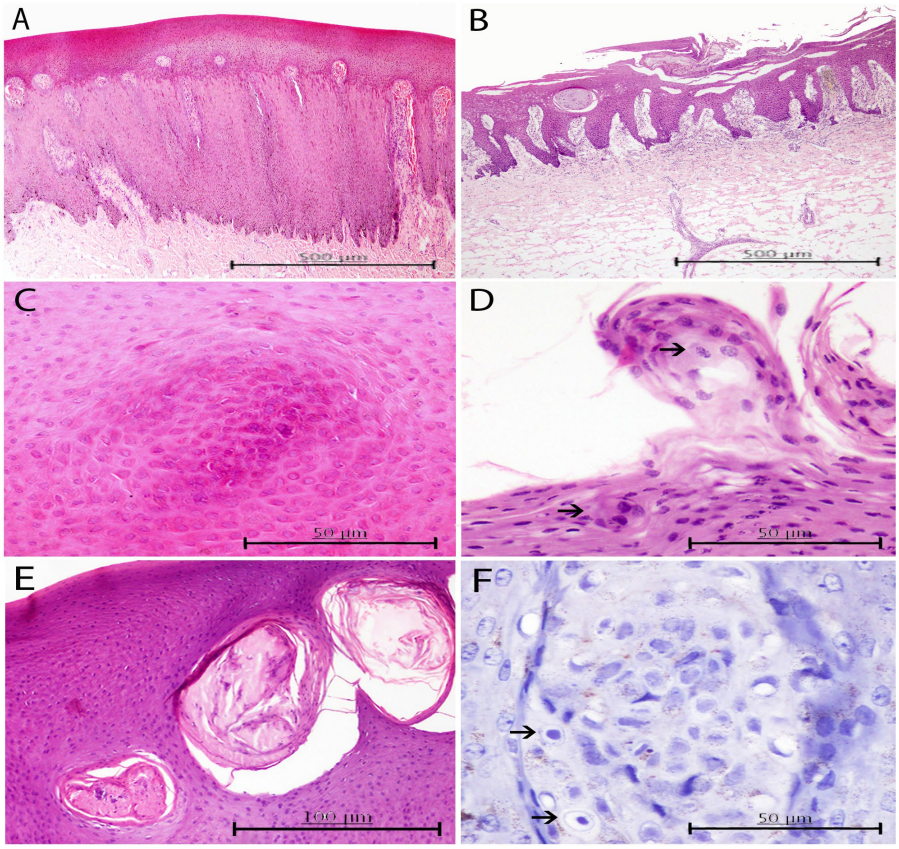

FIGURE 4
Histopathological findings in HV positive skin lesions from three animals of the present study. **(A)** Lesion A2 from case 25. Moderate to marked hyperkeratosis and acanthosis with elongated fused rete ridges that penetrate down to the dermis. Multifocally, some dermal papillae have been occluded due to anastomosing rete ridges, and congestion is observed in the ones remaining uncapped. H and E, ×4. **(B)** Lesion A3 from case 23. Loss of stratum corneum and part of stratum spinosum with the presence of necrotic cellular crusts. H and E, ×4. **(C)** Detailed image of a focal arrangement of acidophilic keratinocytes with ground glass eosinophilic nuclei in stratum spinosum of the same skin lesion. H and E, ×40. **(D)** Lesion A3 from case 23. Round abnormal keratinocytes with condensed nuclei scattered within the upper areas of the stratum (upper arrow). Focal oval-shaped syncytia of basophilic keratinocytes within the intermediate layer (lower arrow). H and E, ×40. **(E)** Lesion A3 from case 28. Multifocal well-delimited oval necrotic areas containing degenerated keratinocytes and neutrophils within the stratum spinosum. H and E, ×20. **(F)** Lesion A2 from case 25. Evidence of INIBs in the most superficial area of a dermal papillae (arrows). Immunochemistry stain. Canine distemper virus (CDV) antibody, ×60.

CePV-1 was also detected in BF, WF, and R lesions, although less frequently. Macroscopically, these skin manifestations can be attributed to poxvirus infection; previous reports have suggested that tattoo-like lesions progress to darker blemishes (persistent stage), turn whiter (regression stage), and become almost invisible (healing stage) (18, 22). The microscopic findings of tattoo-like lesions were observed for the three categories, noting that for BF lesions these histological changes were milder than for WF and R lesions. ICIBs were absent, except for one skin lesion that was coinfected with HV, indicating a possible CePV-1 reactivation. The mild histopathological changes in these macroscopic categories can indicate advanced stages of lesions. Furthermore, all lesions showed high Ct values, which could suggest low viral loads. Together, these findings suggest that the CePV-1-positive skin manifestations may represent chronic stages of the skin disease, thus corroborating these findings with visual diagnostics.

HV was exclusively detected in most gross categories (except the TL-C lesions) across a wide range of skin manifestations, as has been previously reported with wild cetacean populations (45, 71). Furthermore, consistent with previous studies, we commonly observed epidermal necrosis, atypical keratinocytes with both cell and nucleus pleomorphism, and ICI that predominantly involved neutrophils (86, 87). An association between the most prevalent histologic findings and the macroscopic appearance of HV-positive skin lesions was not observed, except for the Ts lesions. Accordingly, all Ts lesions were disrupted in the stratum corneum with well-delimited multifocal crusts of degenerated keratinocytes and neutrophils. Molecular tests revealed that almost all lesions from which HV was identified had high Ct values indicative of low viral loads, suggesting that the lesions could be in chronic or latent stages, though this might also result from poor sampling or nucleic acid degradation. Furthermore, one case in this study showed histopathological changes that were remarkably similar to changes observed in a previously reported HV-positive skin lesion from an Atlantic bottlenose dolphin (34). Both lesions were slightly raised in the stratum corneum, with swollen and irregularly distributed keratinocytes with intranuclear and intracytoplasmic inclusion bodies. In attempting to associate histological changes with the macroscopic appearance of this lesion, Manire and co-workers described the lesion as a hyperplastic area with hundreds of 1–3-mm small spherical firm papules affecting the rostrum, head, dorsal fin, and flanks (34). The lesion in the present study, however, was macroscopically different; it

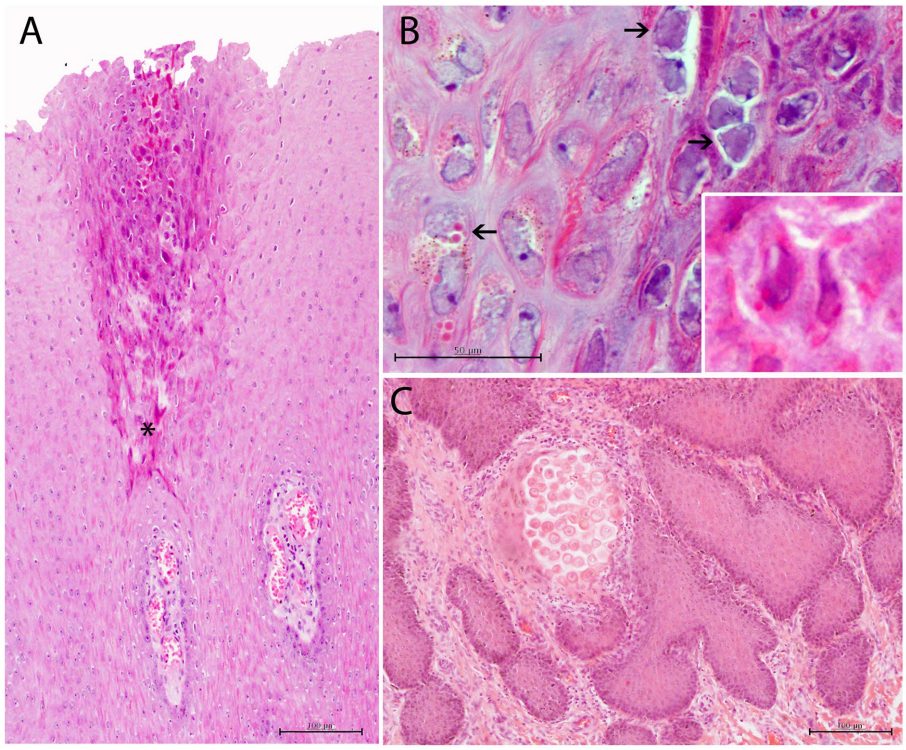

FIGURE 5
Histopathological findings in CePV-1 and HV coinfected skin lesion from case 9. **(A)** Focal irregular arrangement of acidophilic keratinocytes with both basophilic INIBs and small round amphophilic ICIBs in stratum spinosum. Multifocal mild to moderate ICI in dermal papillae. Asterisk indicates the affected area of the stratum spinosum. H and E, ×20. **(B)** Detail of irregular-shaped keratinocytes with small vacuolizations and prominent basophilic INIBs (right upper arrows) and small round pinpoint amphophilic ICIBs (lower left arrow). Lower inset: zoomed-in image of a keratinocyte with both INIBS and ICIBs. H and E, ×60. **(C)** Focal delimited area with abnormal acidophilic necrotic keratinocytes in the basal area of a dermal papilla associated to a combined neutrophilic and eosinophilic ICI. H and E, ×20.

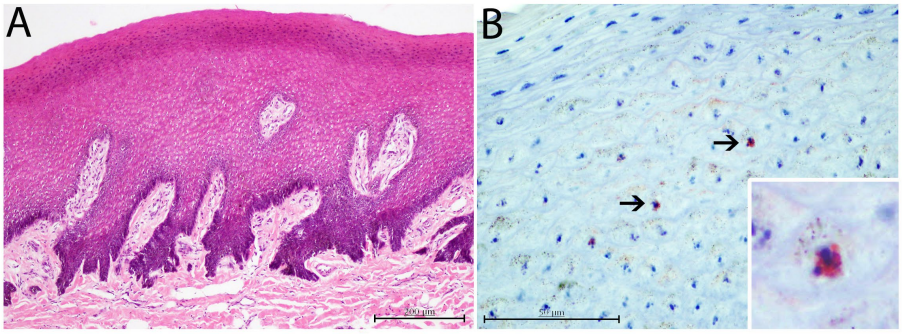

FIGURE 6
Histopathological and immunohistological findings in CeMV positive skin lesion from case 17. **(A)** Mild to moderate diffuse acanthosis with irregular laterally displaced and fused rete ridges. H and E, ×10. **(B)** Slightly immunostained keratinocytes against canine distemper virus (CDV) antibody. Lower inset: zoomed-in image of an immunostained keratinocyte. Immunochemistry stain, ×60.

was a TL-O lesion with an apparent porous consistency localized dorsal to the right eye; CePV-1 was also detected in this lesion.

Six of the nine CePV-1 and HV-coinfected lesions showed tattoo-like patterns. To the best of our knowledge, HV has been detected in various skin manifestations (35, 88), excluding these characteristic lesions that have so far been strictly attributed to CePV, and this study is the first to show HV in tattoo-like lesions. Therefore, the diagnosis of a skin pathogen should therefore use molecular tests to corroborate the results of visual assessments. Histologically, in coinfected lesions, the above-mentioned CePV-1 and HV microscopic findings were more severe, in contrast with lesions from which one of these pathogens was exclusively detected. Molecular tests of coinfected skin lesions often showed variable Ct values, but one of the pathogens usually showed high viral loads. Despite this, the HV-associated microscopic changes were generally more prominent than those associated with CePV-1, which may result from the severe infectiousness of HV in the skin (87, 89). Opportunistic pathogens take advantage of pre-existing wounds as portals of entry (40% of the analyzed lesions in this study derive

TABLE 4 Summary of degree severity of most prevalent histopathological findings by macroscopic categorization of skin lesions of the present study.

Skin associated lesions		Macroscopic classification of skin lesions															
		Tattoo-like oval shaped (*n* = 12)		Tattoo-like coalesced (*n* = 3)		Tattoo-like serpiginous (*n* = 5)		Black-fringed (*n* = 9)		White-fringed (*n* = 8)		Pale (*n* = 1)		Ring (*n* = 6)		Tortuous (*n* = 4)	
		N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Hyperkeratosis	Minimal	1	8.33	0	0	0	0	2	22.22	2	25	0	0	0	0	1	25
	Mild	3	25	0	0	2	40	2	22.22	0	0	1	100	1	16.66	2	50
	Moderate	4	33.33	0	0	2	40	2	22.22	0	0	0	0	0	0	1	25
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		8	**66.66**	**0**	**0**	**4**	**80**	**6**	**66.66**	**2**	**25**	**1**	**100**	**1**	**16.66**	**4**	**100**
Acanthosis	Minimal	1	8.33	1	33.33	0	0	2	22.22	3	37.5	0	0	1	16.66	1	25
	Mild	6	50	0	0	4	80	4	44.44	1	12.5	0	0	1	16.66	3	75
	Moderate	2	16.66	0	0	0	0	2	22.22	0	0	1	100	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		9	**75**	**1**	**33.33**	**4**	**80**	**8**	**88.88**	**4**	**50**	**1**	**100**	**2**	**33.32**	**4**	**100**
Ballooning degeneration	Minimal	1	8.33	1	33.33	2	40	2	22.22	0	0	0	0	0	0	0	0
	Mild	2	16.66	0	0	2	40	0	0	0	0	0	0	1	16.66	0	0
	Moderate	4	33.33	0	0	1	20	0	0	0	0	0	0	0	0	0	0
	Severe	1	8.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		8	**66.66**	**1**	**33.33**	**5**	**100**	**2**	**22.22**	**0**	**0**	**0**	**0**	**1**	**16.66**	**0**	**0**
Spongiosis	Minimal	2	16.66	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mild	1	8.33	0	0	0	0	1	11.11	0	0	0	0	0	0	0	0
	Moderate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Severe	1	8.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		4	**33.33**	**0**	**0**	**0**	**0**	**1**	**11.11**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Necrosis	Minimal	1	8.33	0	0	2	40	0	0	0	0	1	1	0	0	1	25
	Mild	0	0	0	0	0	0	1	11.11	0	0	0	0	0	0	0	0
	Moderate	1	8.33	0	0	1	20	0	0	0	0	0	0	0	0	2	50
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			16.66	**0**	**0**	**3**	**60**	**1**	**11.11**	**0**	**0**	**1**	**100**	**0**	**0**	**3**	**75**
Satellitosis		**1**	**8.33**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**25**
Hyperpigmentation	Minimal	2	16.66	1	33.33	0	0	0	0	1	12.5	1	100	0	0	0	0
	Mild	2	16.66	0	0	0	0	1	11.11	0	0	0	0	0	0	0	0
	Moderate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		4	**33.33**	**1**	**33.33**	**0**	**0**	**1**	**11.11**	**1**	**12.5**	**1**	**100**	**0**	**0**	**0**	**0**
Hypopigmentation	Minimal	0	0	0	0	0	0	0	0	0	0	1	100	0	0	0	0
	Mild	0	0	0	0	0	0	1	11.11	0	0	0	0	0	0	1	25
	Moderate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0	**0**	**0**	**0**	**0**	**0**	**1**	**11.11**	**0**	**0**	**1**	**100**	**0**	**0**	**1**	**25**
Fused rete ridges	Minimal	2	16.66	0	0	0	0	1	11.11	2	25	0	0	0	0	0	0
	Mild	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25
	Moderate	0	0	0	0	0	0	3	33.33	0	0	0	0	0	0	1	25
	Severe	0	0	0	0	0	0	0	0	0	0	1	100	0	0	0	0

(Continued)

TABLE 4 (Continued)

Skin associated lesions		Macroscopic classification of skin lesions															
		Tattoo-like oval shaped (*n* = 12)		Tattoo-like coalesced (*n* = 3)		Tattoo-like serpiginous (*n* = 5)		Black-fringed (*n* = 9)		White-fringed (*n* = 8)		Pale (*n* = 1)		Ring (*n* = 6)		Tortuous (*n* = 4)	
		N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
		2	**16.66**	**0**	**0**	**0**	**0**	**4**	**44.44**	**2**	**25**	**1**	**100**	**0**	**0**	**2**	**50**
ICIBs		**8**	**66.66**	**0**	**0**	**3**	**60**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**16.66**	**0**	**0**
INIBs		**1**	**8.33**	**0**	**0**	0	0	**0**	**0**	**0**	**0**	**1**	**100**	**0**	**0**	**0**	**0**
ICI	Minimal	3	25	1	33.33	1	20	5	55.55	4	50	0	0	1	16.66	2	50
	Mild	4	33.33	0	0	2	40	1	11.11	0	0	0	0	0	0	0	0
	Moderate	0	0	0	0	0	0	2	22.22	0	0	1	100	0	0	2	50
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		7	**58.33**	**1**	**33.33**	**3**	**60**	8	**88.88**	**4**	**50**	**1**	**100**	**1**	**16.66**	**4**	**100**
Congestion	Minimal	3	25	0	0	1	20	2	22.22	2	25	0	0	1	16.66	1	25
	Mild	2	16.66	0	0	1	20	2	22.22	1	12.50	1	100	0	0	0	0
	Moderate	0	0	0	0	3	60	0	0	0	0	0	0	0	0	0	0
	Severe	1	8.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		6	**50**	**0**	**0**	**5**	**100**	**4**	**44.44**	**3**	**37.5**	**1**	**100**	**1**	**16.66**	**1**	**25**
Dyskeratosis/ apoptosis	Minimal	2	16.66	0	0	0	0	4	44.44	0	0	0	0	0	0	0	0
	Mild	2	16.66	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Moderate	1	8.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		5	**41.66**	**0**	**0**	**0**	**0**	**4**	**44.44**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Pearl corns	Minimal	1	8.33	1	33.33	0	0	0	0	1	12.5	0	0	1	16.66	0	0
	Mild	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	25
	Moderate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		1	**8.33**	**1**	**33.33**	**0**	**0**	**0**	**0**	**0**	**12.5**	**0**	**0**	**1**	**16.66**	**1**	**25**

*ICIBs, intracytoplasmic inclusion bodies; INIBs, intranuclear inclusion bodies; *N*, number of lesions.
Bold numbers indicate n and percentage of main histopathological changes observed in each macroscopical category of skin lesion. Higher numbers and percentages are underlined.

from rake marks), and a conceivable pathway of infection in coinfected skin lesions could be the initial entry of CePV-1 leading to the reactivation of latent HV (90, 91). Another possible scenario, although less likely, would be CePV-1 infection as an initial step leading to an increased susceptibility to a secondary HV infection.

CeMV has also been detected in skin lesions, which are related to rash, erosive, and ulcerative patterns (40, 83, 92). The presence of CeMV in skin lesions (1.82%) in this study was low. However, the detection of CeMV in a BF lesion, which can macroscopically be attributed to advanced poxvirus-like lesions, demonstrates the necessity of evidence-based studies to verify pathogens in skin disorders. Additionally, definitive CeMV-related skin patterns have not yet been established in cetaceans. The detection of this re-emergent systemically infectious virus in a skin lesion is important for monitoring cetacean populations to forecast possible epizootic outbreaks. Indeed, the animal in this study with a CeMV-positive lesion also presented multiorgan infection by this same virus.

From the seven CePV-1 sequences used for constructing the phylogenetic tree, three (from the common dolphin, common bottlenose dolphin, and striped dolphin) were mainly clustered according to their detection in the same host species, which is in accordance with previous reports that proposed that the CPV-1 group may contain several sub-groups specific for the different families of odontocetes (49). The other four sequences from our study were non-clustered or were grouped with sequences detected in other host species, possibly because these host species have no entries in GenBank (Risso's dolphin, short-finned pilot whale, and Atlantic spotted dolphin).

On the other hand, the sequences were more widely distributed based on the HV phylogenetic tree, with sequences belonging to both *Gammaherpesvirinae* and *Alphaherpesvirinae* subfamilies. Remarkably, as previously reported (93), herpesviruses seem to be host specific, as most of the sequences in our study were grouped with sequences from the same host species. Only two of the herpesvirus sequences in our study belonged to the *Gammaherpesvirinae* subfamily and showed 100% identity with one detected in a penile lesion in a striped dolphin stranded in the Canary Islands (94). This is consistent with reports of this herpesvirus subfamily, which is more frequently detected in genital and mucosal lesions (95), though it has also been detected in the skin (35, 96).

Only three alphaherpesvirus sequences in this study were grouped with sequences previously obtained from skin lesions (86, 96), while most of them were close to sequences acquired from other tissues including ovary (97), pulmonary lymph node (27, 31), kidney, lung, spleen (26), and brain (30). This suggests that the same strains probably affect tissues other than skin. In this sense, another distinct alphaherpesvirus sequence was detected from the adrenal gland of a bottlenose dolphin (case 27, CET 1151), which in turn presented four skin lesions with two different alphaherpesvirus strains. The amplicon recovered from the adrenal gland showed a 100% similarity to a sequence obtained from the skin of a stranded bottlenose dolphin in Germany (86). Moreover, this amplicon was highly similar to another identified from a skin lesion of the same animal, which suggests that the virus may have been disseminated (25).

Finally, one of the skin lesions from this study that histologically presented large intranuclear inclusion bodies surrounded by a clear halo was similar to sequences from animals with HV-related acute and severe lesions including INIBs, necrotic changes, malacia, and lymphoid depletion. Likewise, Eva Sierra and co-workers (30) identified sequences from four cases presenting with severe acute brain lesions that could lead to death; these sequences clustered with the abovementioned pathogenic HV strains. However, as stated above, caution should be exercised when interpreting these short sequences.

5. Conclusion

In light of the growing emergence of viral diseases in cetacean populations, methods other than visual assessment are needed to diagnose skin diseases and enable their use as potential health indicators. For this purpose, stranded cetaceans are outstanding resources for testing evidence-based approaches to identifying viruses from skin lesions. Future studies should combine macroscopic and histopathological studies of skin lesions with quantitative molecular analyses to further understand the epidemiology of viral skin diseases in cetacean wild populations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The requirement of ethical approval was waived by Environmental department of the Canary Islands Government and the Spanish Ministry of Environment for the studies involving animals because no experiments were performed on live animals. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

ES, AF, CF, and MAn: conceptualization and review and editing. SS-G, ES, AF, MAr, MAB, CF, IF-J, and AC-R: methodology and formal analysis. SS-G, ES, and CF: writing – original draft preparation. ES, AF, MAr, CF, and MAB: supervision. AF, ES, and MAr: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1188105/full#supplementary-material

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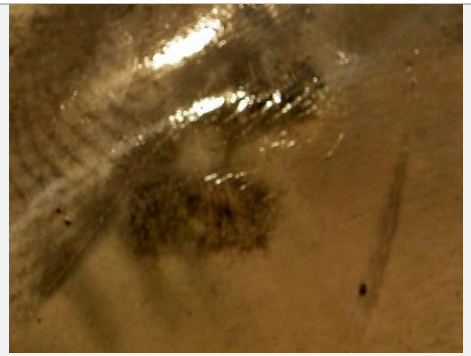
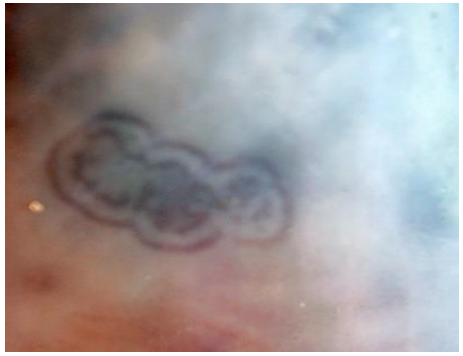
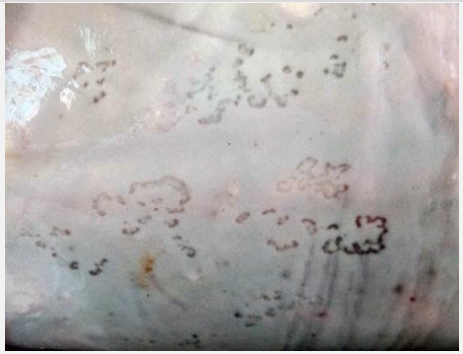
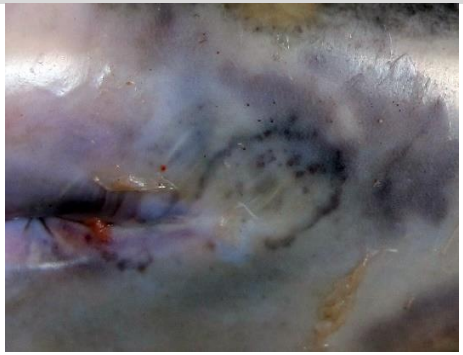
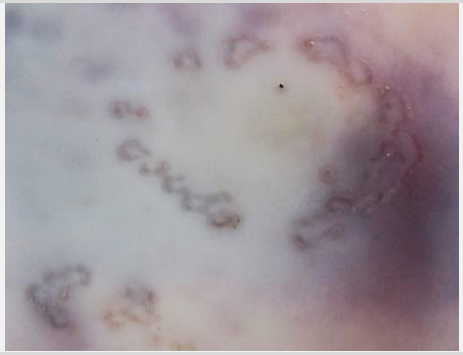
Supplementary Table 1 Biological and stranding conditions of the 31 cetaceans included in the present study.

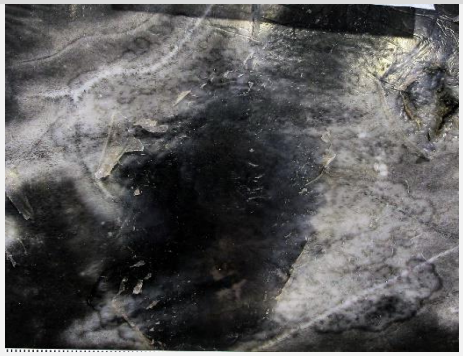
CASE N.	ID CODE	SPECIES	AGE	SEX	SD	SL	SS	DC	CM
1	CET 566	*S. coeruleoalba*	A	F	26/03/2011	Tenerife	D	2	F
2	CET 601	*S. frontalis*	J	M	05/02/2012	Tenerife	D	2	RT
3	CET 642	*S. frontalis*	A	F	01/02/2013	Lanzarote	A	1	RT
4	CET 663	*D. delphis*	A	F	06/05/2013	Tenerife	A	2	RT
5	CET 705	*S. coeruleoalba*	J	F	23/03/2014	Gran Canaria	A	1	R
6	CET 748	*S. coeruleoalba*	J	M	06/03/2015	Lanzarote	A	2	RT
7	CET 751	*G. griseus*	A	F	16/03/2015	Tenerife	D	4	F
8	CET 947	*D. delphis*	J	M	02/01/2019	Fuerteventura	D	4	F
9	CET 951	*S. coeruleoalba*	C	M	23/01/2019	La Palma	D	2	F
10	CET 959	*S. coeruleoalba*	A	M	19/02/2019	Fuerteventura	D	4	F
11	CET 969	*G. macrorhynchus*	C	M	24/03/2019	Tenerife	A	1	RT
12	CET 983	*S. coeruleoalba*	J	M	20/04/2019	Gran Canaria	D	2	F
13	CET 984	*G. griseus*	C	M	26/04/2019	Gran Canaria	A	1	RT
14	CET 985	*S. coeruleoalba*	A	M	27/04/2019	Tenerife	D	2	RT
15	CET 991	*S. coeruleoalba*	A	F	09/05/2019	Fuerteventura	D	2	R
16	CET 995	*G. macrorhynchus*	J	M	20/05/2019	Gran Canaria	D	2	R
17	CET 1020	*T. truncatus*	J	F	09/08/2019	Tenerife	D	2	RT
18	CET 1035	*S. coeruleoalba*	J	F	04/10/2019	Fuerteventura	A	2	F
19	CET 1044	*S. frontalis*	C	F	05/12/2019	Tenerife	D	4	RT
20	CET 1045	*D. delphis*	A	F	05/12/2019	Fuerteventura	A	2	F
21	CET 1056	*S. frontalis*	C	M	24/01/2020	Tenerife	D	2	RT
22	CET 1058	*S. frontalis*	C	M	27/01/2020	Gran Canaria	D	2	F
23	CET 1067	*S. frontalis*	C	F	12/03/2020	Tenerife	D	2	F
24	CET 1069	*S. coeruleoalba*	A	M	13/03/2020	Gran Canaria	D	2	F
25	CET 1103	*T. truncatus*	C	M	13/06/2020	Gran Canaria	A	2	RT
26	CET 1138	*S. frontalis*	C	M	18/12/2020	Gran Canaria	D	2	F
27	CET 1151	*T. truncatus*	C	M	21/02/2021	Tenerife	D	2	RT
28	CET 1152	*S. frontalis*	C	M	26/02/2021	Gran Canaria	A	1	F
29	CET 1153	*D. delphis*	A	M	02/03/2021	Gran Canaria	D	2	F
30	CET 1173	*S. frontalis*	J	F	17/04/2021	Tenerife	D	2	RT
31	CET 1181	*G. macrorhynchus*	C	F	05/12/2021	Tenerife	D	2	RT

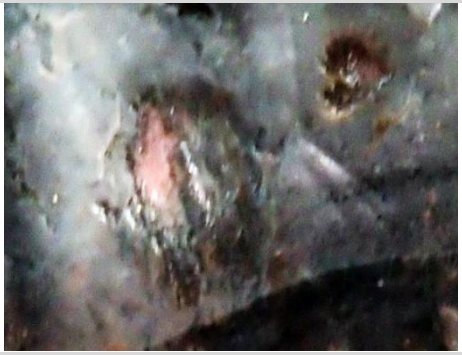
*AGE (A = adult, J = juvenile, C = calf, N = neonate); SEX (F = female, M = male); SD (stranding date); SL (stranding location); SS (stranding stage: A = alive; D = dead); DC (decomposition code); CM (conservation method); DC (1 = extremely fresh carcass, 2 = fresh carcass, 3 = moderate decomposition, 4 = advanced decomposition, and 5 = mummified or skeletal remains); CM (F = frozen, RT = room temperature

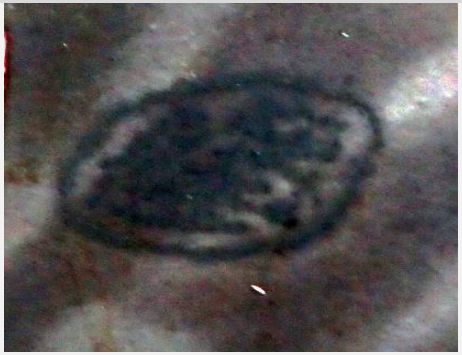
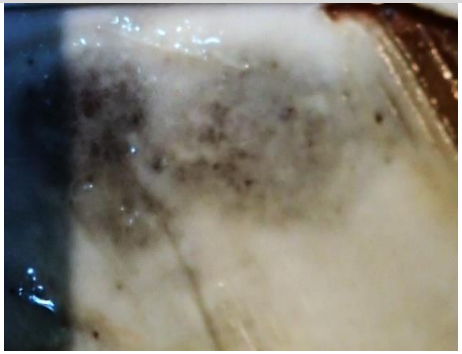
Supplementary Table 2 Gross classification and histopathology of the 55 skin lesions with their respective molecular results from the 31 animals of the present study.

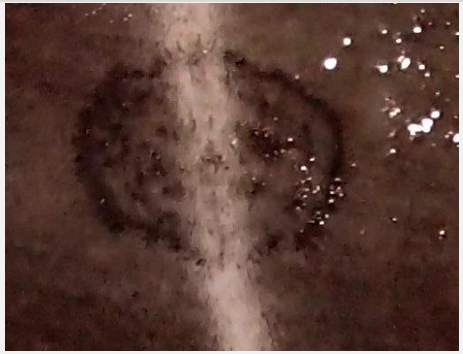
CASE N.	ID CODE	MACROSCOPIC CLASSIFICATION		HISTOPATHOLOGICAL FINDINGS	MOLECULAR RESULTS	ASSOCIATED RAKE MARKS/WOUNDS
1	CET 566	A1	 WHITE - FRINGED	NE	Negative	Yes
2	CET 601	A1*	 TATTOO-LIKE COALESCED	Minimal hyperkeratosis, acanthosis, and ballooning degeneration. Mild intracellular edema.	CePV-1+ (ON600451)	No

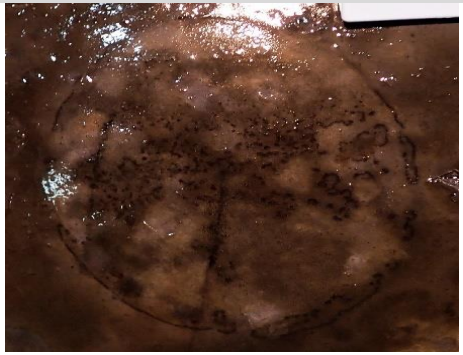
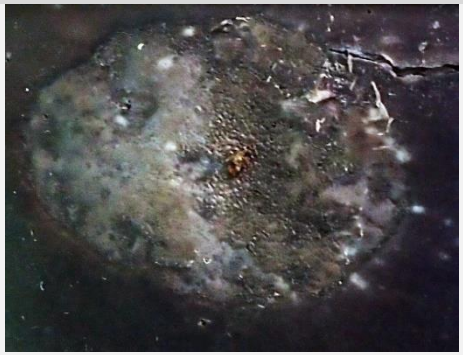
3	CET 642	A1	 TATTOO-LIKE SERPIGINOUS	Minimal necrosis. Mild acanthosis, intracellular edema, and inflammatory cell infiltration. Moderate hyperkeratosis, ballooning degeneration and congestion. Presence of ICIBs.	CePV-1+ (ON600452); HV+ (OM456331)	No
4	CET 663	A1	 TATTOO-LIKE OVAL SHAPE	NE	CePV-1+ (ON600453)	No
5	CET 705	A1		Minimal ballooning degeneration and intracellular edema. Mild hyperkeratosis and acanthosis. Moderate congestion. Presence of ICIBs.	CePV-1+ (ON600454)	No

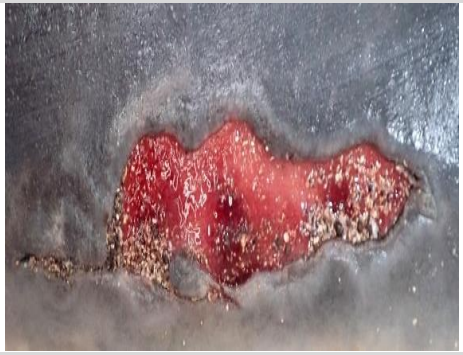
			TATTOO-LIKE SERPIGINOUS			
6	CET 748	A1	 TATTOO-LIKE SERPIGINOUS	NE	CePV-1+ (ON600455)	Yes
7	CET 751	A1	 TATTOO-LIKE OVAL SHAPE	Minimal spongiosis, hyperpigmentation, fused rete ridges, inflammatory cell infiltration and dyskeratosis/apoptosis. Mild acanthosis. Moderate hyperkeratosis, ballooning degeneration and intracellular edema.	CePV-1+ (ON600456)	Yes

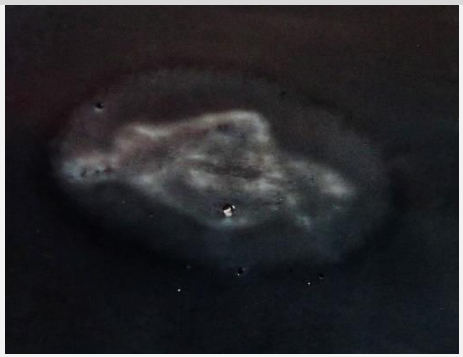
8	CET 947	A3	 TATTOO-LIKE OVAL SHAPE	NE	CePV-1+ (ON600460); HV+ (OM456332)	Yes
9	CET 951	A1	 TATTOO-LIKE OVAL SHAPE	Minimal presence of pearl corns. Mild inflammatory cell infiltration and congestion. Moderate acanthosis, necrosis, and dyskeratosis/apoptosis. Presence of ICIBs and INIBs. Presence of satellitosis.	CePV-1+ (ON600461); HV+ (OM456333)	No
10	CET 959	A1		Minimal inflammatory cell infiltration	Negative	Yes

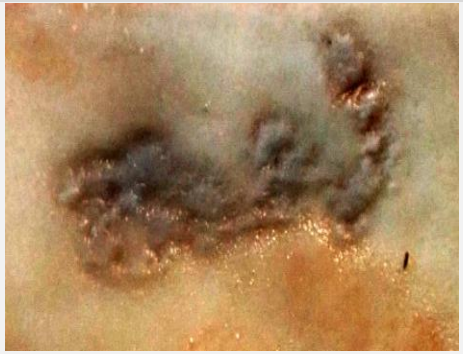
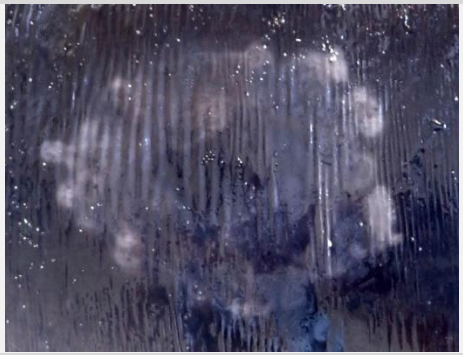
			ULCERATIVE			
11	CET 969	A6	 TATTOO-LIKE OVAL SHAPE	Minimal intracellular edema, inflammatory cell infiltration and congestion. Mild hyperkeratosis, acanthosis, and ballooning degeneration. Presence of ICIBs.	CePV-1+ (ON600462)	No
12	CET 983	A3	 TATTOO-LIKE COALESCED	Minimal acanthosis, hyperpigmentation, inflammatory cell infiltration and presence of pearl corns.	CePV-1+ (ON600463)	Yes

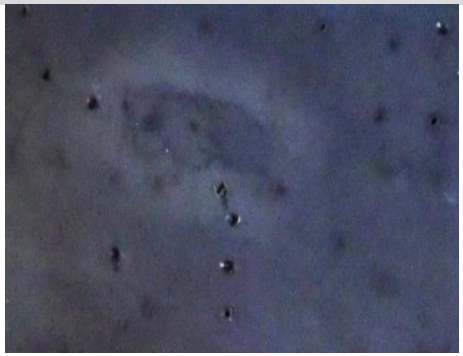
13	CET 984	A4	 TATTOO-LIKE OVAL SHAPE	NA	CePV-1+ (ON600464); HV+ (OM456334)	Yes
14	CET 985	A1	 TATTOO-LIKE SERPIGINOUS	Minimal necrosis. Mild hyperkeratosis, acanthosis, ballooning degeneration and intracellular edema. Moderate congestion. Presence of ICIBs.	CePV-1+ (ON600465); HV+ (OM456335)	Yes
15	CET 991	A3		Absence of associated histopathological changes	Negative	Yes

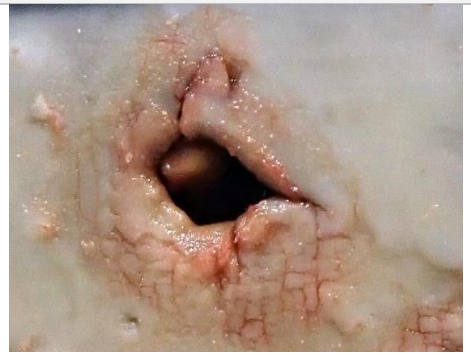
			RING			
16	CET 995	A1	 TATTOO-LIKE OVAL SHAPE	Minimal hyperkeratosis and ballooning degeneration. Mild acanthosis, spongiosis, inflammatory cell infiltration and dyskeratosis/apoptosis. Severe congestion.	CePV-1+ (ON600457)	Yes
17	CET 1020	A1	 TATTOO-LIKE OVAL SHAPE	Minimal spongiosis, necrosis, fused rete ridges and inflammatory cell infiltration. Mild hyperkeratosis and acanthosis, and dyskeratosis/apoptosis. Moderate ballooning degeneration and intracellular edema. Presence of ICIBs.	CePV-1+ (ON600466); HV+ (OM456336)	No

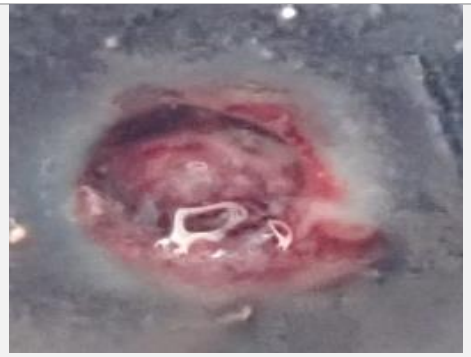
18	CET 1035	A2	 BLACK-FRINGED	Minimal hyperkeratosis and inflammatory cell infiltration. Mild acanthosis. Moderate fused rete ridges.	CeMV+ (ON314830)	Yes
19	CET 1044	A1	 RING	NA	HV+ (OM456337)	Yes
20	CET 1045	A4		NA	HV+ (OM456338)	Yes

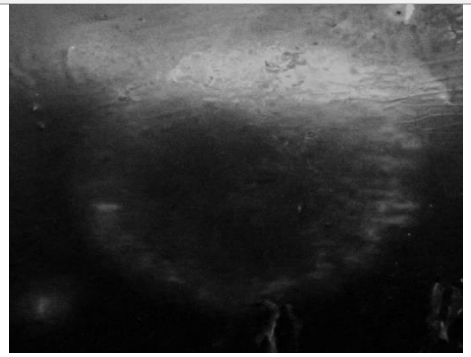
			ULCERATIVE			
21	CET 1056	A1	 BLACK-FRINGED	Minimal fused rete ridges and inflammatory cell infiltration. Mild hypopigmentation and congestion. Moderate acanthosis.	HV+ (OM456339)	No
22	CET 1058	A1	 BLACK-FRINGED	Minimal ballooning degeneration, intracellular edema, and congestion. Mild inflammatory cell infiltration. Moderate necrosis.	CePV-1+ (ON600467); HV+ (OM456340)	No

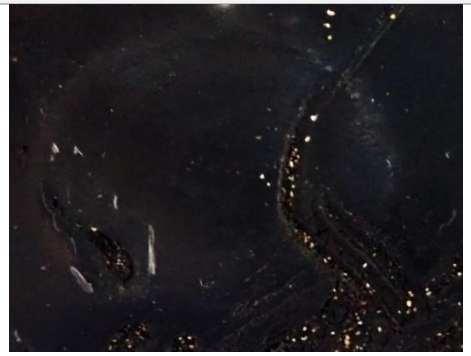
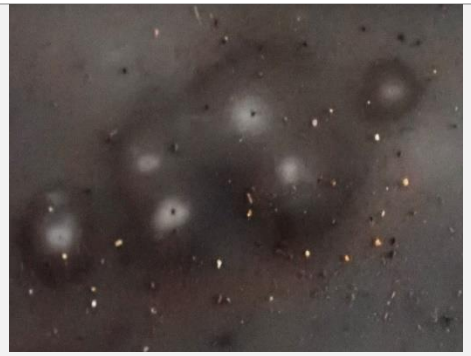
23	CET 1067	A3	 TORTUOUS	Mild acanthosis, hypopigmentation, and presence of pearl corns. Moderate hyperkeratosis, necrosis, fused rete ridges and inflammatory cell infiltration. Presence of ICIBs.	HV+ (ON314829)	No
24	CET 1069	A1	 RING	NE	CePV-1+ (ON600468)	Yes
25	CET 1103	A2*		Minimal necrosis, hyperpigmentation, and hypopigmentation. Mild hyperkeratosis, intracellular edema, and congestion. Moderate acanthosis and inflammatory cell infiltration. Severe fused rete ridges.	HV+ (OM456341)	No

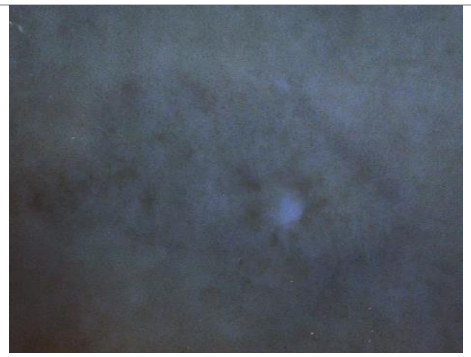
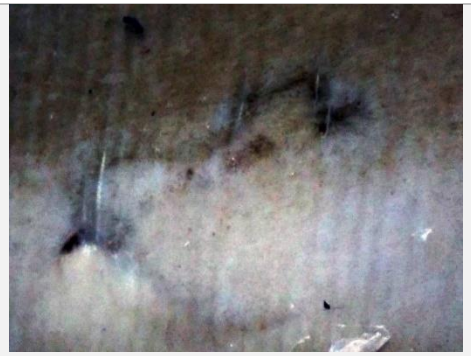
			PALE	Presence of INIBs.		
26	CET 1138	A1	 WHITE-FRINGED	NE	HV+ (OM456342)	Yes
		A2	 WHITE-FRINGED	NA	HV+ (OM456342)	Yes

		A3	 TARGET-LIKE	NE	HV+ (OM456342)	Yes
		A4	 TARGET-LIKE	NE	Negative	Yes
		A5	 ULCERATIVE	NE	HV+ (OM456342)	No

27	CET 1151	A1*	Image rights not deserved. *Image previously reported in: "Segura-Göthlin, S., A. Fernández, M. Arbelo, I. Felipe-Jiménez, A. Colom-Rivero, J. Almunia, and E. Sierra, 2021: The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean Poxvirus. Anim. 2021, Vol. 11, Page 2814* ***11****, 2814, DOI: 10.3390/ANI11102814".* TATTOO-LIKE SERPIGINOUS	Minimal intracellular edema and fused rete ridges. Mild acanthosis, ballooning degeneration and congestion. Moderate hyperkeratosis. Presence of ICIBs.	CePV-1+ (ON600458); HV+ (OM456343)	No
		A3	 TARGET-LIKE	NA	HV+ (OM456344)	Yes
		A4	 ULCERATIVE	NA	HV+ (OM456343)	No
		A6*	Image rights not deserved. *Image previously reported in: "Segura-Göthlin, S., A. Fernández, M. Arbelo, I. Felipe-Jiménez, A. Colom-Rivero, J. Almunia, and E. Sierra, 2021: The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean*	Minimal intracellular edema and pearl corns.	CePV-1+ (ON600458); HV+ (OM456344)	No

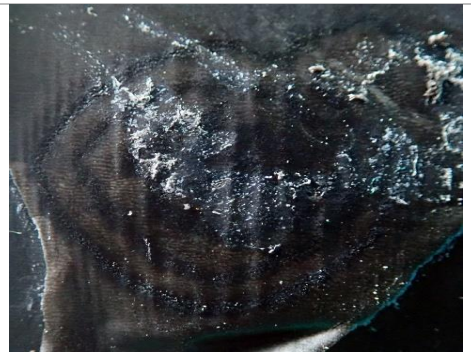
			Poxvirus. Anim. 2021, Vol. 11, Page 2814 ***11****, 2814, DOI: 10.3390/ANI11102814".* RING	Mild hyperkeratosis, ballooning degeneration and acanthosis. Presence of ICIBs.		
28	CET 1152	A1	 TORTUOUS	Minimal hyperkeratosis, acanthosis, necrosis, and inflammatory cell infiltration.	HV+ (OM456345)	No
		A2	 WHITE-FRINGED	Minimal hyperkeratosis, necrosis, fused rete ridges, inflammatory cell infiltration, congestion, and pearl corns. Mild acanthosis.	HV+ (OM456345)	No

		A3	 TORTUOUS	Mild hyperkeratosis and acanthosis. Moderate necrosis and inflammatory cell infiltration.	Negative	No
		A4	 RING	Minimal acanthosis, intracellular edema, inflammatory cell infiltration and congestion.	HV+ (OM456345)	Yes
		A5	 BLACK-FRINGED	Minimal intracellular edema and dyskeratosis/apoptosis. Mild hyperkeratosis, acanthosis, and necrosis. Moderate inflammatory cell infiltration.	HV+ (OM456345)	No

29	CET 1153	A1	 BLACK-FRINGED	Minimal inflammatory cell infiltration. Moderate hyperkeratosis, acanthosis, and fused rete ridges.	Negative	No
		A2	 TORTUOUS	Minimal inflammatory cell infiltration and congestion. Mild hyperkeratosis, acanthosis, and fused rete ridges.	Negative	No
		A3	 BLACK-FRINGED	Minimal acanthosis, inflammatory cell infiltration, congestion, and dyskeratosis/apoptosis. Moderate fused rete ridges.	Negative	No

30	CET 1173	A1*	Image rights not deserved. *Image previously reported in: "Segura-Göthlin, S., A. Fernández, M. Arbelo, I. Felipe-Jiménez, A. Colom-Rivero, J. Almunia, and E. Sierra, 2021: The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean Poxvirus. Anim. 2021, Vol. 11, Page 2814* ***11****, 2814, DOI: 10.3390/ANI11102814".* TATTOO-LIKE OVAL SHAPE	Minimal congestion. Mild acanthosis, intracellular edema, hyperpigmentation, and inflammatory cell infiltration. Moderate hyperkeratosis and ballooning degeneration. Presence of ICIBs.	CePV-1+ (ON600459)	No
		A2*		Minimal congestion. Mild acanthosis, intracellular edema, hyperpigmentation, and inflammatory cell infiltration. Moderate hyperkeratosis and ballooning degeneration. Presence of ICIBs.	CePV-1+ (ON600459)	No
		A3*		Minimal congestion. Mild acanthosis, intracellular edema, hyperpigmentation, and inflammatory cell infiltration. Moderate hyperkeratosis and ballooning degeneration. Presence of ICIBs.	CePV-1+ (ON600459)	No
		A4*	Image rights not deserved. *Image previously reported in: "Segura-Göthlin, S., A. Fernández, M. Arbelo, I. Felipe-Jiménez, A. Colom-Rivero, J. Almunia, and E. Sierra, 2021: The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean Poxvirus. Anim. 2021, Vol. 11, Page 2814* ***11****, 2814, DOI: 10.3390/ANI11102814".* BLACK-FRINGED	Minimal acanthosis, ballooning degeneration, intracellular edema, congestion, and dyskeratosis/apoptosis. Mild hyperkeratosis, spongiosis, and inflammatory cell infiltration.	CePV-1+ (ON600459)	No

		A5*		Minimal ballooning degeneration and dyskeratosis/apoptosis. Mild acanthosis, hyperpigmentation, and congestion. Moderate hyperkeratosis and inflammatory cell infiltration.	CePV-1+ (ON600459)	No
		A6*	Image rights not deserved. *Image previously reported in: "Segura-Göthlin, S., A. Fernández, M. Arbelo, I. Felipe-Jiménez, A. Colom-Rivero, J. Almunia, and E. Sierra, 2021: The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean Poxvirus. Anim. 2021, Vol. 11, Page 2814* ***11****, 2814, DOI: 10.3390/ANI11102814".* WHITE-FRINGED	Minimal hyperkeratosis, acanthosis, hyperpigmentation, and inflammatory cell infiltration.	CePV-1+ (ON600459)	No
		A7*		Minimal acanthosis, inflammatory cell infiltration, and congestion.	CePV-1+ (ON600459)	No
		A8*	Image rights not deserved. *Image previously reported in: "Segura-Göthlin, S., A. Fernández, M. Arbelo, I. Felipe-Jiménez, A. Colom-Rivero, J. Almunia, and E. Sierra, 2021: The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean Poxvirus. Anim. 2021, Vol. 11, Page 2814 11, 2814, DOI: 10.3390/ANI11102814".* RING	NA	CePV-1+ (ON600459)	No
		A9*	Image rights not deserved. *Image previously reported in: "Segura-Göthlin, S., A. Fernández, M. Arbelo, I. Felipe-Jiménez, A. Colom-Rivero, J. Almunia, and E. Sierra, 2021: The Validation of a Non-Invasive Skin Sampling Device for Detecting Cetacean Poxvirus. Anim. 2021, Vol. 11, Page 2814 11, 2814, DOI: 10.3390/ANI11102814".* WHITE-FRINGED	Minimal acanthosis, fused rete ridges, and inflammatory cell infiltration. Mild congestion.	CePV-1+ (ON600459)	No
		A10*		NA	CePV-1+ (ON600459)	No

31	CET 1181	A1	 TATTOO-LIKE OVAL SHAPE	Minimal acanthosis and dyskeratosis/apoptosis. Mild hyperkeratosis and ballooning degeneration. Presence of ICIBs.	CePV-1+ (ON600469)	No
		A2	 TATTOO-LIKE COALESCED	Minimal ballooning degeneration.	Negative	No
		A3	 BLACK-FRINGED	Minimal hyperkeratosis, acanthosis, and inflammatory cell infiltration.	CePV-1+ (ON600469)	No

*CePV, cetacean poxvirus; HV, herpesvirus; CeMV, cetacean morbillivirus; A1 – A10 = skin lesion samples 1 to 10; NA, not applicable; NE, not evaluated; +, positive; -, negative. Asterisks indicate positive cases that have been previously published.

Supplementary Table 3. Percentage of identity of the sequences from the present study with the closest available ones in GenBank.

CASE N.	ID CODE	SKIN LESION	SEQUENCES											
			CePV						HV					
			Accession Number	Bp	Organism	Percentage Identity	Isolation Source	References	Accession Number	Bp	Organism	Percentage Identity	Isolation Source	References
1	CET 566	A1	-	-	-	-	-	-	-	-	-	-	-	-
2	CET 601	A1	ON600451	497	CePV-1	93.75% (OQ102395)	Skin	NA	-	-	-	-	-	-
3	**CET 642**	**A1**	**ON600452**	**497**	**CePV-1**	**93.75% (OQ102395)**	**Skin**	**NA**	**OM456331**	**193**	**Alphaherpesvirus**	**98.45% (MG437205)**	**NA**	**NA**
4	CET 663	A1	ON600453	497	CePV-1	100% (KC409046)	Skin	(1)	-	-	-	-	-	-
5	CET 705	A1	ON600454	497	CePV-1	99.80% (KC409049)	Skin	(1)	-	-	-	-	-	-
6	CET 748	A1	ON600455	497	CePV-1	99.80% (KC409049)	Skin	(1)	-	-	-	-	-	-
7	CET 751	A1	ON600456	497	CePV-1	95.97% (KC409049)	Skin	(1)	-	-	-	-	-	-
8	**CET 947**	**A3**	**ON600460**	**98**	**CePV-1**	**96.91% (MH005249)**	**Skin**	(2)	**OM456332**	**193**	**Alphaherpesvirus**	**100% (MG437205)**	**NA**	**NA**
9	**CET 951**	**A1**	**ON600461**	**85**	**CePV-1**	**90% (AY463006)**	**Skin**	(3)	**OM456333**	**193**	**Alphaherpesvirus**	**97.41% (MG437205)**	**NA**	**NA**
10	CET 959	A1	-	-	-	-	-	-	-	-	-	-	-	-
11	CET 969	A6	ON600462	98	CePV-1	96.91% (MH005249)	Skin	(2)	-	-	-	-	-	-
12	CET 983	A3	ON600463	98	CePV-1	96.91 (MH005249)	Skin	(2)	-	-	-	-	-	-
13	**CET 984**	**A4**	**ON600464**	**98**	**CePV-1**	**93.81% (MH005249)**	**Skin**	**(2)**	**OM456334**	**181**	**Alphaherpesvirus**	**100% (KP995683)**	**Brain**	**(4)**
14	**CET 985**	**A1**	**ON600465**	**98**	**CePV-1**	**96.91% (MH005249)**	**Skin**	**(2)**	**OM456335**	**169**	**Gammaherpesvirus**	**100% (KM248274)**	**Penis**	**(5)**
15	CET 991	A3	-	-	-	-	-	-	-	-	-	-	-	-
16	CET 995	A1	ON600457	497	CePV-1	94.96% (KC409049)	Skin	(1)	-	-	-	-	--	-
17	**CET 1020**	**A1**	**ON600466**	**98**	**CePV-1**	**96.94% (MH005249)**	Skin	(2)	**OM456336**	**194**	**Alphaherpesvirus**	**96.91% (MG437205)**	**NA**	**NA**
18	CET 1035	A2	-	-	-	-	-	-	-	-	-	-		-
19	CET 1044	A1	-	-	-	-	-	-	OM456337	190	Alphaherpesvirus	100% (MN179657)	Brain	NA

20	CET 1045	A4	-	-	-	-	-	-	OM456338	181	Alphaherpesvirus	100% (MN179655)	Brain	NA
21	CET 1056	A1	-	-	-	-	-	-	OM456339	190	Alphaherpesvirus	100% (MN179657)	Brain	NA
22	**CET 1058**	**A1**	**ON600467**	**82**	**CePV-1**	**92.68% (AY952950)**	**Skin**	(3)	**OM456340**	**193**	**Alphaherpesvirus**	**98.45% (MG437205)**	**NA**	**NA**
23	CET 1067	A3	-	-	-	-	-	-	ON314829	169	Gammaherpesvirus	98.42% (KM248274)	Penis	(5)
24	CET 1069	A1	ON600468	75	CePV-1	92.11% (AY952950)	Skin	(3)	-	-	-	-	-	-
25	CET 1103	A2	-	-	-	-	-	-	OM456341	190	Alphaherpesvirus	100% (MG437217)	NA	NA
26	CET 1138	A1	-	-	-	-	-	-	OM456342	190	Alphaherpesvirus	99.47% (MN179657)	Brain	NA
		A2												
		A3												
		A4							-	-	-	-	-	-
		A5							OM456342	190	Alphaherpesvirus	99.47% (MN179657)	Brain	NA
27	CET 1151	**A1**	**ON600458**	**497**	**CePV-1**	**100% (OQ102395)**	**Skin**	**-.**	**OM456343**	**190**	**Alphaherpesvirus**	**99.47% (AY949832)**	**Skin**	**(6)**
		A3	-	-	-	-	-	-	OM456344	190	Alphaherpesvirus	100% (AY608707)	Skin	(6)
		A4							OM456343	190	Alphaherpesvirus	99.47% (AY949832)	Skin	(6)
		A6	**ON600458**	**497**	**CePV-1**	**100% (OQ102395)**	**Skin**	**-**	**OM456344**	**190**	**Alphaherpesvirus**	**100% (AY608707)**	**Skin**	**(6)**
28	CET 1152	A1	-	-	-	-	-	-	OM456345	193	Alphaherpesvirus	98.45% (MG437205)	NA	NA
		A2							OM456345					
		A3							-	-	-	-	-	-
		A4							OM456345	193	Alphaherpesvirus	98.45% (MG437205)	NA	NA
		A5							OM456345					
29	CET 1153	A1	-	-	-	-	-	-	-	-	-	-	-	-
		A2												
		A3												
30	CET 1173	A1	ON600459	497	CePV-1	98.99% (MF458199)	Skin	-	-	-	-	-	-	-
		A2												

		A3													
		A4													
		A5													
		A6													
		A7													
		A8													
		A9													
		A10													
31	CET 1181	A1	ON600469	77	CePV-1	93.51% (AY952950)	Skin	(3)	-	-	-	-	-	-
		A2	-	-	-	-	-	-						
		A3	ON600469	77	CePV-1	93.51% (AY952950)	Skin	(3)						

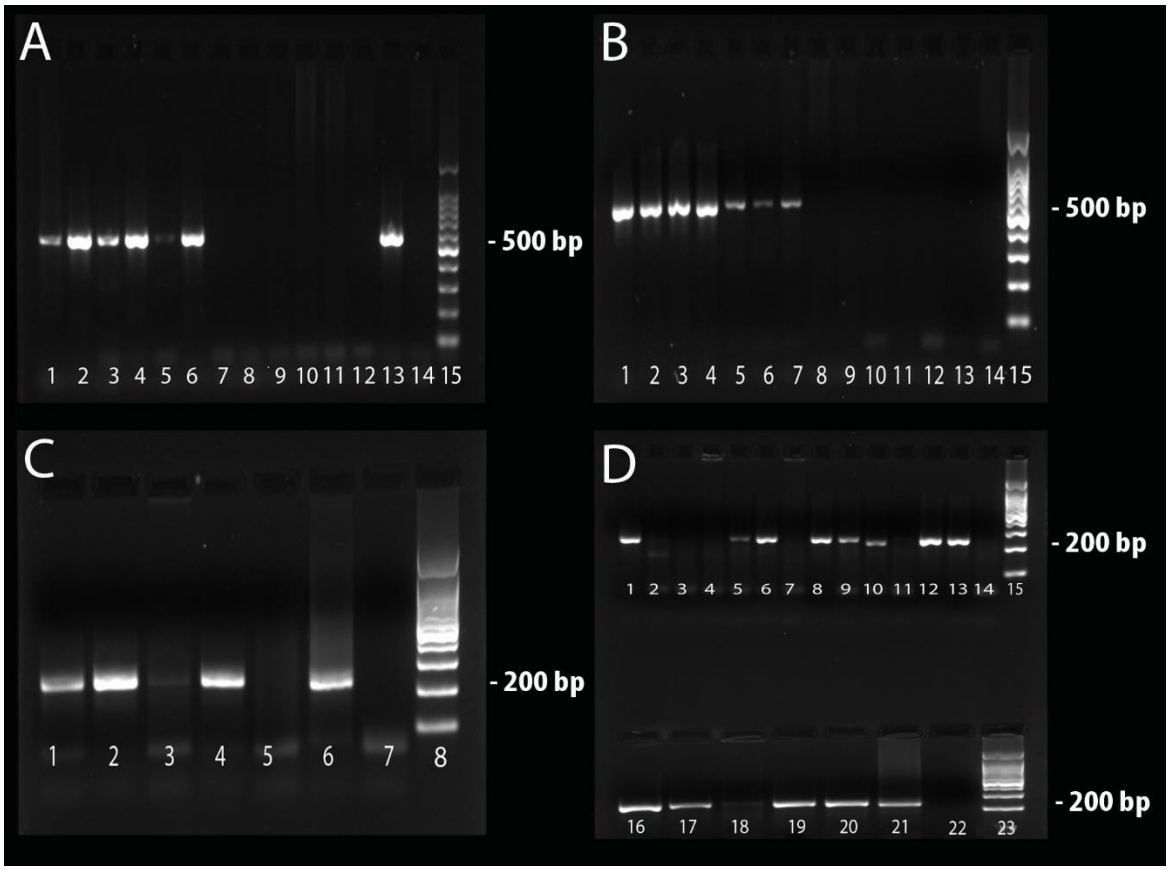
*Bp, base pairs; CePV-1, cetacean poxvirus 1; HV, herpesvirus; NA, not available; -, absent.

Supplementary Table 4 Most prevalent histologic findings in skin lesions of the present study.

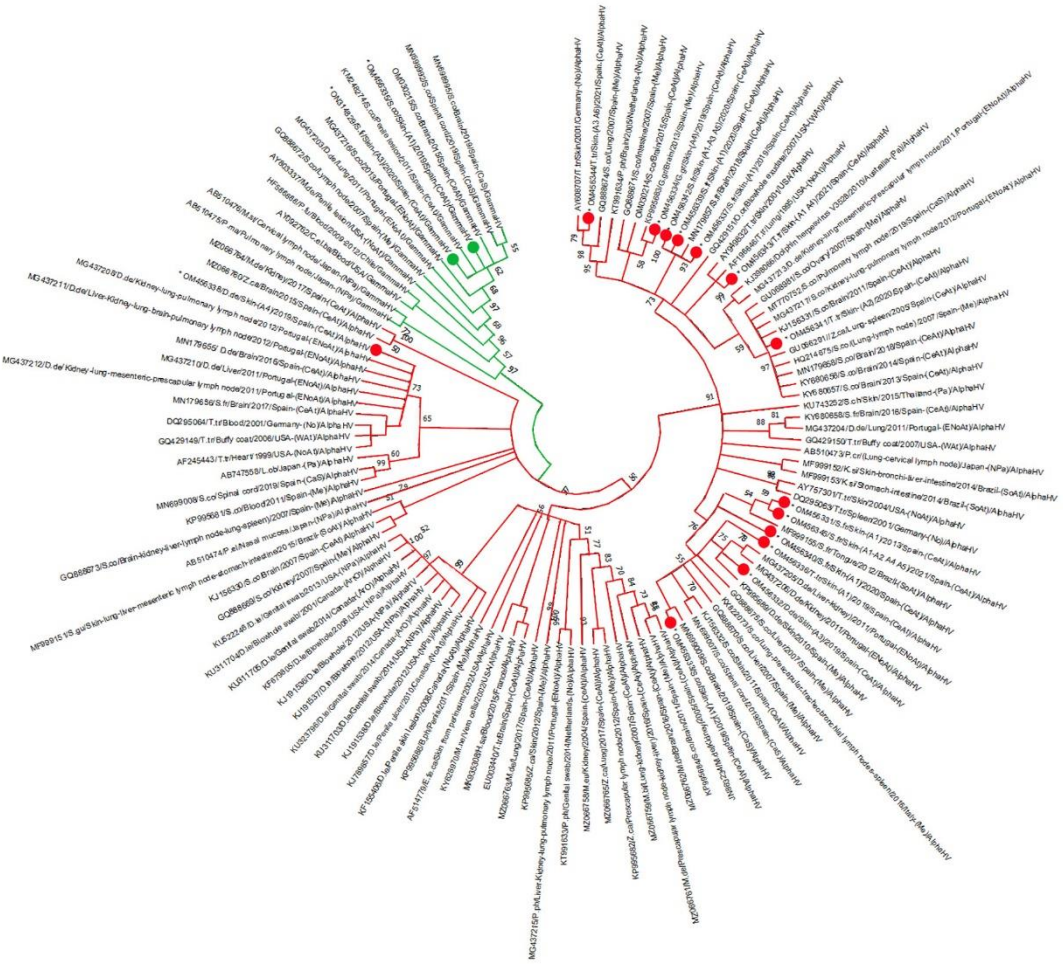
CASE N.	ID CODE	LESION	MC	MOST PREVALENT HISTOLOGICAL FINDINGS														
				Hk	At	BD	Sp	IE	Ne	St	Hp	Ho	FRR	ICIBs	INIBs	ICI	Cg	Dk/Ap	PC
2	CET 601	A1	TL-C	+	+	+	-	++	-	no	-	-	-	no	no	-	-	-	-
3	CET 642	A1	TL-S	+++	++	+++	-	++	+	no	-	-	-	yes	no	++	+++	-	-
5	CET 705	A1	TL-S	++	++	+	-	+	--	-	-	-	-	no	no	-	+++	-	-
7	CET 751	A1	TL-O	+++	++	+++	+	+++	-	no	+	-	+	yes	no	+	-	+	-
9	CET 951	A1	TL-O	-	+++	-	-	-	+++	yes	-	-	-	yes	yes	++	++	+++	+
10	CET 959	A1	U	-	-	NE	NE	NE	-	no	-	-	-	no	no	+	-	-	-
11	CET 969	A6	TL-O	++	++	++	-	+	-	no	-	-	-	yes	no	+	+	-	-
12	CET 983	A3	TL-C	NE	+	NE	NE	NE	-	no	+	-	-	no	no	+	-	-	+
14	CET 985	A1	TL-S	++	++	++	-	++	+	no	-	-	-	yes	no	-	+++	-	-
15	CET 991	A3	R	NE	-	-	-	-	-	no	-	-	-	no	no	-	-	-	-
16	CET 995	A1	TL-O	+	++	+	++	NE	-	no	-	-	-	no	no	++	++++	++	-
17	CET 1020	A1	TL-O	++	++	+++	+	+++	+	no	-	-	+	yes	no	+	-	++	-
18	CET 1035	A2	BF	+	++	NE	NE	NE	-	no	-	-	+++	no	no	+	-	-	-
21	CET 1056	A1	BF	-	+++	-	-	-	-	no	-	++	+	no	no	+	++	-	-
22	CET 1058	A1	BF	NE	NE	+	-	+	+++	no	-	-	-	no	no	++	+	-	-
23	CET 1067	A3	Ts	+++	++	NE	NE	NE	+++	yes	-	++	+++	no	no	+++	-	-	++
25	CET 1103	A2	P	++	+++	-	-	++	+	no	+	+	++++	no	yes	+++	++	-	-
27	CET 1151	A1	TL-S	+++	++	++	-	+	-	-	-	-	+	yes	no	+	++	-	-
		A6	R	++	++	++	-	+	-	no	-	-	-	yes	no	-	-	-	+
28	CET 1152	A1	Ts	+	+	-	-	-	+	no	-	-	-	no	no	+	-	-	-
		A2	WF	+	++	-	-	-	+	no	-	-	+	no	no	+	+	-	+
		A3	Ts	++	++	-	-	-	+++	no	-	-	-	no	no	+++	-	-	-
		A4	R	-	+	-	-	+	-	no	-	-	-	no	no	+	+	-	-

		A5	BF	++	++	-	-	+	++	no	-	-	-	no	no	+++	-	+	-
29	CET 1153	A1	BF	+++	+++	-	-	-	-	no	-	-	+++	no	no	+	-	-	-
		A2	Ts	++	++	-	-	-	-	no	-	-	++	no	no	+	+	-	-
		A3	BF	-	++	-	-	-	-	no	-	-	+++	no	no	+	+	+	-
30	CET 1173	A1	TL-O	+++	++	+++	-	++	-	no	++	-	-	yes	no	++	+	-	-
		A2	TL-O	+++	++	+++	-	++	-	no	++	-	-	yes	no	++	+	-	-
		A3	TL-O	+++	+++	++++	++++	+++	-	no	+	-	-	yes	no	-	++	-	-
		A4	BF	++	+	+	+	+	-	no	-	-	-	no	no	++	+	+	-
		A5	BF	+++	++	+	+	-	-	no	++	-	-	no	no	+++	++	+	-
		A6	WF	+	+	-	-	-	-	no	+	-	-	no	no	+	-	-	-
		A7	WF	NE	+	-	-	-	-	no	-	-	-	no	no	+	+	-	-
		A9	WF	NE	+	-	-	-	-	no	-	-	+	no	no	+	++	-	-
31	CET 1181	A1	TL-O	++	+	++	-	-	-	no	-	-	-	yes	no	-	-	+	-
		A2	TL-C	-	-	+	-	-	-	no	-	-	-	no	no	+	-	-	-
		A3	BF	+	+	-	-	-	-	no	-	-	-	no	no	+	-	-	-

*Hk, hyperkeratosis; At, acanthosis; Hp, hyperplasia; BD, ballooning degeneration; Sg, spongiosis; IE, intracytoplasmic oedema; MC, macroscopic classification; Nc, necrosis; St, satellistosis; Hp; hyperpigmentation; Ho, hypopigmentation; FRR, fused rete ridges; ICIBs, inclusion bodies; INIBs, intranuclear inclusion bodies; ICI, inflammatory cell infiltration; Cg, congestion; Dk/Ap, dyskeratosis/apoptosis; PC, pearl corns; A1 – A9 = skin lesion samples 1 to 9; BF (black-fringed); R (ring); T (target-like); Ts (tortuous); TL-C (tattoo-like, coalesced); TL-S (tattoo-like, serpiginous); TL-O (tattoo-like, oval-shaped); P (pale); U (ulcerative); WF (white-fringed); NE, not evaluable; -, absent; +, minimal; ++, mild; +++, moderate; ++++, severe.



Supplementary Figure 1. Poxvirus and herpesvirus positive samples in agarose gel 2%. (**A, B**): Positive poxvirus results using conventional PCR. (**A**): Line 1: case 2; line 2: case 3; line 3: case 4; line 4: case 5; line 5: case 6; line 6: case 7; line 13: case 16; line 15: molecular-weight size marker. (**B**): Line 1 and 3: case 27; line 2 and 4: case 27; line 5: case 30; line 6: case 30; line 7: case 30; line 15: molecular-weight size marker. (**C, D**): agarose gel electrophoresis of HV q-PCR of positive samples. (**C**): Line 1: case 8; line 2: case 9; line 3: case 27; line 4: case 27; line 6: PCR positive control; line 8: molecular-weight size marker. (**D**): Line 1: case 3; line 5: case 17; line 6: case 19; line 8: case 21; line 9: case 23; line 10: case 25; line 12: case 26 (skin lesion A2); line 13: case 26 (skin lesion A3); line 15: molecular-weight size marker; line 16: case 26 (skin lesion A5); line 17: case 27 (lesion A1); line 18: case 27 (skin lesion A3); line 19: case 27 (skin lesion A4); line 20: case 28 (skin lesion A1); line 21: case 28 (skin lesion A4); line 23: molecular-weight size marker.



Supplementary Figure 2. 99 HV nucleotide-sequence phylogenetic tree.

4.

CONCLUSIONS

1. Stranded cetaceans offer the opportunity for pilot studies such as the replication of novel sampling protocols. Likewise, it has been proven that cytological cell samplers are a reliable technique for sampling tattoo-like skin lesions, obtaining enough genetic material to detect CePV, presenting a similar or even better efficacy than skin biopsies depending on the genomic extraction method used.

2. Through the use of cytological cell samplers, it is possible to detect CePV in a non-invasive way reducing the impact on the welfare of cetaceans under human care. Its practicality, time efficiency in obtaining samples and suitability for use by either veterinarians or caregivers make this device a potential sampling alternative to skin biopsies.

3. Cytological cell samplers achieve, for the first time in cetaceans, the detection of CePV in both tattoo-like skin lesions and in apparently healthy skin from individuals under human care who have not previously exhibited clinical signs of skin disease.

4. The detection in cetaceans in human care from two different zoos of the same CePV sequence with high homology to sequences previously obtained from wild cetaceans from the North and South Atlantic may mean that the virus could have been transmitted between individuals for years since the introduction of the first wild cetaceans in zoos and aquariums.

5. The study of a wide range of skin lesions and the molecular analysis of pathogens such as CePV, HV and CeMV has allowed us to improve our knowledge of the pathogenesis of these emerging diseases. Thus, it has been possible to detect for the first time the presence of HV in lesions that until now have been exclusively associated with CePV and, in addition, the high prevalence of these viral agents in skin lesions has been verified.

6. The gross and histopathological characterization of the skin lesions together with the molecular detections obtained has made it possible to differentiate lesions associated both with CePV and HV specifically, as well as lesions

associated with both agents. For example, macroscopic lesions with a tortuous pattern could be associated with HV.

7. The exceptional detection in this study of the comorbidity of CePV and HV in the same skin lesions raises new questions about the interaction of these pathogens and their effects on the infection and pathogenesis of one on the other.

5.
RESUMEN EXTENDIDO

5.1 INTRODUCCIÓN

Hasta la fecha, la piel de los cetáceos se ha considerado una herramienta prometedora para obtener una mejor compresión y amplio conocimiento acerca de estos mamíferos marinos, convirtiéndose, por lo tanto, en el objeto de investigación de una amplia gama de campos científicos (Schick et al., 2013; Barratclough et al., 2019a). Entre la abundante información que puede proporcionar, la piel de los cetáceos es un elemento clave para la evaluación del estado de salud tanto de los animales como del ecosistema acuático (Wilson et al., 1999; Van Bressem et al., 2009; Mouton y Botha, 2012; Barlow et al., 2019). Es por ello que los estudios a largo plazo sobre la piel de estos mamíferos marinos permiten identificar posibles amenazas antropogénicas en la naturaleza, como la presencia de contaminantes tóxicos (Aubail et al., 2013; Mancia et al., 2018; Zanuttini et al., 2019; Baini et al., 2020), los efectos del tráfico marino o las interacciones con las actividades pesqueras, entre otros (Van Bressem et al., 2007; Butterworth, 2017; Leone et al., 2019; Fossi et al., 2020; Puig-Lozano et al., 2020; Womersley et al., 2021). Podría decirse que la exposición continuada a todas estas situaciones estresantes puede llegar a provocar inmunosupresión y una mayor susceptibilidad a enfermedades infecciosas afectando, en primera instancia, a la piel actuando como vía de entrada de los agentes biológicos (Van Bressem et al., 2009; Bossart, 2011; Miller et al., 2018; Koch et al., 2018; Barratclough et al., 2019b; Bossart et al., 2019).

En este sentido, las enfermedades de la piel juegan un papel esencial en la obtención de un conocimiento aproximado del estado general de estos mamíferos marinos, siendo su estudio en relación con la salud de los cetáceos lo más documentado hasta el momento (Maldini et al., 2010; Mouton y Botha, 2012; Romero y Keith, 2012; Gonzalvo et al., 2014; Nelson et al., 2015). Existe una considerable diversidad de microorganismos oportunistas que afectan a la piel de los cetáceos, generalmente aprovechando áreas ya previamente dañadas para penetrar y causar infección (Mouton y Botha, 2012; Bartlett et al., 2016). Por ejemplo, las infecciones cutáneas bacterianas y fúngicas secundarias suponen a menudo una complicación en las heridas cutáneas de los cetáceos, especialmente en aquellas que están ulceradas, empeorando, y a veces, dificultando considerablemente el proceso de curación de la piel. Además, esto último combinado con los impactos antropogénicos provoca consecuencias negativas en la salud de estos mamíferos marinos, afectando considerablemente las funciones biológicas de los animales (Higgins, 2000; Melero et al., 2011; Esperón et al., 2012; Ueda et al., 2013; Bossart et al., 2019; Duignan et

al., 2020). Sin embargo, lo más documentado hasta ahora en relación con microorganismos oportunistas es el aumento de la frecuencia de enfermedades víricas de la piel, cuyo desarrollo a lo largo del tiempo proporciona una visión de la salud del hospedador y de las respuestas inmunológicas tanto a nivel individual como de toda la población (Barr et al., 1989; Smolarek et al., 2006; Barnett et al., 2015; Fiorito et al., 2015; Dagleish et al., 2021).

Entre las infecciones virales que afectan a la piel, “Tattoo Skin Disease” (TSD) ha sido significativamente una de las más reportadas en muchas especies de cetáceos debido a que se distingue visualmente de otras lesiones de la piel (Harzen y Brunnick, 1997; Van Bressem et al., 2003; Fury y Reif, 2012; Hart et al., 2012). Esto se debe a que lo que se ha reportado hasta ahora sobre las manifestaciones cutáneas de las infecciones por poxvirus en cetáceos muestran una apariencia característica ampliamente conocida como "tattoo" (tatuajes), que se corresponde con lesiones con centro plano o ligeramente deprimido con un patrón punteado en el interior y márgenes negros bien delimitados (Geraci et al., 1979; Bossart y Duignan, 2018; Powell et al., 2018a). Dependiendo de su estadio evolutivo, estas lesiones cutáneas pueden oscurecerse o aparecer completamente pálidas, perdiendo sus márgenes. A pesar de que hasta la fecha no se ha demostrado que esta enfermedad vírica tenga sustancialmente la capacidad patogénica de causar la muerte de los cetáceos infectados, se considera que su aparición está relacionada con la salud y/o sistema inmune de los individuos afectados como consecuencia de la incapacidad de éstos para enfrentarse correctamente a un entorno marino en constante desequilibrio (Van Bressem et al., 2003; Fury y Reif, 2012; Cocumelli et al., 2018; Koch et al., 2018). De acuerdo con esto último, es distintivo que las lesiones de tatuaje evolucionen y persistan en la piel de los cetáceos de forma independiente e indefinida, así como que desaparezcan para reaparecer posteriormente de forma recurrente (Geraci et al., 1979; Van Bressem et al., 1999; Mouton y Botha, 2012; Fiorito et al., 2016; Powell et al., 2020). Debido a todo lo anterior, el poxvirus puede considerarse un indicador de salud general potencialmente útil en esos mamíferos marinos (Van Bressem et al., 2009; Van Bressem et al., 2015).

A pesar de estar ampliamente descritos, la información genética que existe sobre estos virus está muy limitada como para clasificar correctamente los poxvirus que infectan a estos mamíferos marinos. Publicaciones anteriores sugieren que pertenecen a un género no asignado de la subfamilia *Chordopoxvirinae*, siendo clasificados tentativamente en un nuevo género conocido como poxvirus de cetáceos (CePV), que incluye al menos dos subgrupos: poxvirus de cetáceos 1 (CePV-1) que infecta odontocetos, y poxvirus de cetáceos 2 (CePV-2), que infecta misticetos (Bracht et al., 2006; Blacklaws et al., 2013; Rodrigues et

al., 2020). La escasa secuenciación de este agente vírico se debe principalmente a la considerable facilidad para distinguir su manifestación cutánea tan característica, lo que lleva al intento inconsistente de relacionar la presencia del patógeno en estas lesiones sin realizar métodos diagnósticos para corroborar la infección (Bearzi et al., 2009; Van Bressem et al., 2017; Powell et al., 2018b; Stylos, 2019). En consecuencia, aparte de que esto puede llevar a suposiciones con falta de base científica, limita el conocimiento de si el CePV puede estar presente en otras manifestaciones cutáneas distintas a las lesiones "tattoo" o si otros patógenos concomitantes pueden estar asociados a estas lesiones (Melero et al., 2014). Son pocos los estudios que han desarrollado métodos de diagnóstico sensibles para detectar con éxito CePV, siendo las diferentes técnicas de PCR (para los genes ADN polimerasa y ADN topoisomerasa I) consideradas la aproximación diagnóstica por excelencia para su detección (Blacklaws et al., 2013; Cocumelli et al., 2018; Sacristán et al., 2018; Sacristán et al., 2018; Luciani et al., 2022). Complementariamente, la descripción de cuerpos de inclusión intracitoplasmáticos acidófilos en células epidérmicas mediante histología, así como la identificación de partículas virales por microscopía electrónica de transmisión, suelen realizarse con el fin de servir como procedimientos de apoyo que mejoren y refuercen el diagnóstico (Sacristán et al., 2018; Luciani et al., 2022). De cualquiera de las maneras, para realizar las técnicas mencionadas se requieren muestras de piel de lesiones cutáneas sospechosas de estar infectadas por CePV.

Generalmente, las biopsias de piel son el método de elección para tomar muestras de piel de lesiones cutáneas en cetáceos (Parsons y Durban, 2003; Gales et al., 2009; Romero y Keith, 2012; Noren y Mocklin, 2012). En el medio salvaje, las técnicas de toma de biopsias a distancia se han desarrollado y utilizado en múltiples estudios en las últimas décadas, normalmente manejadas con ballestas o rifles modificados, ambos con puntas de muestreo y dardos de acero inoxidable (Krutzen et al., 2002; Sinclair et al., 2015). Sin embargo, la accesibilidad a los cetáceos en libertad en el medio marino es logísticamente compleja, requiere mucho tiempo y es relativamente cara, lo que hace que el muestreo de piel sea muy difícil de llevar a cabo en muchas situaciones (Gales et al., 2009; Romero y Keith, 2012; de Mello y de Oliveira, 2016; Boggs et al., 2019). Además de esto último, no existen evidencias claras sobre el posible impacto negativo que estos métodos de muestreo pueden suponer en cetáceos salvajes, habiéndose reportado, por ahora, comportamientos aberrantes durante la persecución y el muestreo, y algunos casos de alteración en la cicatrización de heridas (Gauthier y Sears, 1999; Bearzi, 2000; Cantor et al., 2010; Kiszka et al., 2010; Tezanos-Pinto y Baker, 2012; Noren y Mocklin, 2012). Alternativamente, los

cetáceos varados proporcionan otra fuente valiosa para adquirir conocimientos significativos sobre estos mamíferos marinos (Arbelo et al., 2013; Díaz-Delgado et al., 2018). De este modo, sus cadáveres representan un acceso directo e ilimitado para tomar muestras representativas, así como recoger biopsias de piel y, por tanto, evaluar enfermedades que puedan afectar a este órgano sin riesgo de comprometer el bienestar de los individuos. Correspondientemente, la mayoría de lo documentado hasta ahora sobre CePV se ha conseguido gracias a esta alternativa, habiéndose identificado esta enfermedad de la piel en cetáceos de todo el mundo, desde el mar Atlántico (Geraci et al., 1979; Fiorito et al., 2015) y el océano Pacífico (Van Bressem et al., 1993; Van Bressem y Van Waerebeek, 1996; Bracht et al., 2006), hasta el Mar del Norte (Blacklaws et al., 2013), afectando a un amplio número de especies. Su distribución geográfica es tal que también se ha comprobado la aparición de esta enfermedad cutánea en cetáceos bajo cuidado humano, concretamente en delfines mulares (*Tursiops truncatus*) (Ridgway, 1984; Van Bressem et al., 2017; St Leger et al., 2018).

En relación con esto último, debido a la creciente preocupación social del mantenimiento de estos mamíferos marinos en zoológicos y acuarios, ha habido un auge en el estudio del bienestar de los cetáceos en estas instituciones (Brando et al., 2018; Clegg et al., 2021; Lauderdale et al., 2021). Hoy en día, este concepto se ha convertido en una prioridad para los zoológicos modernos, a pesar de que gran parte del estudio sobre el bienestar animal se ha realizado en animales de granja (Salas et al., 2018). Sin embargo, los métodos y enfoques utilizados en la evaluación del bienestar de los animales de granja se han adaptado y aplicado en animales de otros ámbitos, incluidos los que se encuentran en zoológicos (Clegg et al., 2015). El bienestar animal tiene una naturaleza compleja, estando compuesto por varios factores como la salud física y fisiológica, el estado emocional y el comportamiento (Broom, 1991; Carenzi y Verga, 2009; Manteca, 2012). Debido a su heterogeneidad, el estudio del bienestar animal es un reto, lo que lleva a la mayoría de las investigaciones a centrarse en uno de los aspectos mencionados o en el uso combinado de distintas variables cualitativas que podrían estar basadas en "recursos" (aspectos ambientales) o en "animales" (aspectos conductuales, físicos y fisiológicos) (Clegg et al., 2017; von Fersen et al., 2018; Wolfensohn et al., 2018).

En consecuencia, el enfoque común para evaluar el bienestar de los animales en los zoológicos consiste en análisis cualitativos a través de indicadores basados con la salud física (Manteca et al., 2016; Whitham et al., 2017). De esta manera, la prevalencia e incidencia de enfermedades y lesiones en piel se ha propuesto recientemente como un indicador

plausible de salud en relación con el bienestar en cetáceos (Clegg et al., 2015). En consecuencia, las lesiones cutáneas por CePV, además de haber sido consideradas como un potencial indicador de salud, también pueden comportarse como una variable para evaluar el bienestar, sirviendo para identificar posibles desequilibrios tanto en el medio ambiente como en la salud del hospedador que puedan estar relacionados con su aparición. Por lo tanto, la fácil accesibilidad de los cetáceos bajo cuidado humano puede proporcionar el conocimiento, las habilidades y los recursos para entender mejor la dinámica huésped-patógeno de CePV y su efecto sobre la salud y el bienestar de los cetáceos. Además, su estudio en estas condiciones también permitiría extrapolar los hallazgos adquiridos en cetáceos de poblaciones salvajes.

A pesar de las ventajas mencionadas anteriormente, existen pocas publicaciones científicas sobre el CePV en cetáceos bajo cuidado humano en comparación con cetáceos salvajes. Las razones de esto podrían estar relacionadas con lo mencionado anteriormente sobre la posible aflicción física que pueden suponer las biopsias de piel. En instalaciones bajo cuidado humano, esta técnica puede considerarse una manipulación de larga duración que podría implicar la restricción física de los animales, además del hecho de que es muy probable que no se pueda asegurar el mantenimiento del bienestar de los individuos durante todo el procedimiento. A esto se le suma el hecho de que el empleo de esta técnica de muestreo para evaluar, repetidamente, enfermedades cutáneas en cetáceos bajo cuidado humano podría suscitar dudas éticas, especialmente cuando existen otros métodos de muestreo menos invasivos que podrían utilizarse para intentar determinar su eficacia en el diagnóstico de la enfermedad (Amos et al., 1992; Valsecchi et al., 1998; Gendron y Mesnick, 2001; Wang y Maibach, 2011).

En consecuencia, esta tesis doctoral se desarrolló para ampliar el conocimiento, en la medida de lo posible, de la patogénesis y epidemiología del CePV y otros posibles patógenos que pueden estar asociados tanto a lesiones tipo “tattoo” como a muchas otras lesiones diferentes en cetáceos varados en las costas canarias, así como en delfines mulares comunes mantenidos en dos instalaciones en las Islas Canarias. Por lo tanto, los objetivos específicos fueron

1. Desarrollar y probar la viabilidad de un dispositivo de muestreo cutáneo no invasivo para detectar poxvirus de cetáceos comparándolo con biopsias cutáneas en un estudio piloto realizado en cetáceos varados.

2. Aplicar esta técnica de muestreo cutáneo no invasivo en cetáceos mantenidos en zoológicos y acuarios para muestrear lesiones similares a tatuajes sin comprometer su bienestar, sirviendo como un procedimiento de muestreo rápido, adaptable a cuidadores y entrenadores de dichas instituciones, y como un dispositivo alternativo a las biopsias cutáneas.

3. Emplear técnicas de PCR como métodos de diagnóstico sostenibles para determinar patógenos asociados tanto en lesiones tipo “tattoo” y como en otras, así como contribuir a ampliar la limitada información genómica de poxvirus de cetáceos.

4. Caracterizar histopatológicamente lesiones cutáneas específicas y correlacionarlas con hallazgos macroscópicos y moleculares de patógenos seleccionados (poxvirus, herpesvirus y morbillivirus).

5.2. PUBLICACIONES CIENTÍFICAS

5.2.1 VALIDACIÓN DE UN DISPOSITIVO DE MUESTREO CUTÁNEO NO INVASIVO PARA LA DETECCIÓN DE POXVIRUS DE LOS CETÁCEOS.

La mayoría de los estudios relacionados con el CePV consisten en análisis fotográficos y en la identificación visual de lesiones "tattoo", presuponiendo que su aparición se debe a la infección de la piel por parte de este patógeno. La falta de métodos de diagnóstico para corroborar la presencia del virus en estas lesiones puede dar lugar a conjeturas incoherentes. Para ello, es fundamental la toma de biopsias cutáneas de lesiones tipo "tattoo" y su posterior análisis molecular, que es el método diagnóstico de elección para confirmar la infección por CePV. Sin embargo, cuando se trata de cetáceos bajo cuidado humano, deberían realizarse técnicas de muestreo alternativas a las biopsias cutáneas para mejorar los procedimientos de manipulación y, de esta manera, contemplar la ética animal, considerando, por tanto, la opción del muestreo de piel de forma no invasiva. Sin embargo, actualmente no se ha demostrado que se consigan resultados viables en la posterior detección molecular del patógeno tras el empleo de otros métodos de recogida distintos a las biopsias cutáneas.

Esta investigación consistió en un estudio piloto realizado en doce lesiones tipo "tattoo" de dos cetáceos varados en las costas canarias, que fueron recolectadas tanto a través de biopsias como con cepillos citológicos, con el fin de comparar la fiabilidad de este último dispositivo, con el futuro objetivo de que pueda ser reproducible en cetáceos bajo cuidado humano como método alternativo a las biopsias. Para ello, se llevaron a cabo dos procedimientos diferentes de extracción genómica (DNA Tissue Kit S™ (QuickGene, Kurabo, Japón) y DNeasy™ Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA)) con todas las muestras, con el objetivo de asociar cuál de ellos da mejores resultados al comparar el método de recolección. La detección molecular de CePV se realizó mediante PCR a tiempo real. Como resultado, se obtuvieron tasas de positividad del 91,7% y 83,3% con biopsias y cepillos citológicos mediante el método de extracción Quickgene, respectivamente, frente a la tasa del 100% utilizando cepillos citológicos y el método de extracción Qiagen. En conclusión, los cepillos citológicos son un dispositivo de muestreo no invasivo fiable para obtener suficiente material genético para la detección de CePV en lesiones cutáneas tipo "tattoo" y que podría ser reproducible en cetáceos bajo cuidado humano.

5.2.2 HACIA LA COMPRENSIÓN DE LA DINÁMICA HUÉSPED-PATÓGENO DE POXVIRUS EN CETÁCEOS: ENFOQUE ALCANZABLE MEDIANTE LA APLICACIÓN DE UN MUESTREO CUTÁNEO NO INVASIVO REPETITIVO EN DELFINES MULARES (TURSIOPS TRUNCATUS) BAJO CUIDADO HUMANO

Los zoológicos y acuarios, dentro de sus tres principios fundamentales (conservación, educación e investigación) buscan mantener a los cetáceos bajo cuidado humano en sus mejores estándares de salud y bienestar. Con este fin, además de estar constantemente reinventando, mejorando y asegurando la actualización de las distintas formas de evaluación de la salud animal, estas instituciones proporcionan la oportunidad de desarrollar investigaciones basadas en evidencias científicas con el fin de lograr una mejor comprensión de la salud de los cetáceos. Entre ellos, recientemente se ha propuesto que el estado físico de los cetáceos sea un recurso animal de fácil acceso para evaluar el estado de salud general de estos mamíferos marinos.

De esta manera, esta investigación se enfocó en el estudio de la piel de los cetáceos, concretamente en la enfermedad llamada "Tattoo Skin Disease", TSD, mejorando técnicas de muestreo a través de un dispositivo no invasivo, sirviendo como un enfoque innovador para mejorar la gestión y el manejo de estos animales. Así, a lo largo del año 2019, se llevó a cabo una recolección repetitiva de piel aparentemente sana y con lesiones tipo "tattoo" de 18 delfines mulares alojados en dos instalaciones (Instalación 1, FAC1 e Instalación 2, FAC2) en las Islas Canarias a través del empleo de cepillos citológicos con el fin de detectar CePV.

El presente estudio ha servido para demostrar que los cepillos citológicos consisten en un dispositivo práctico no invasivo asertivo en la detección de CePV a partir de piel desprendida. Del mismo modo, también se logró una detección excepcional del patógeno en piel sana no sólo en un grupo social en el que diferentes individuos mostraban la enfermedad cutánea (FAC2), sino también en un grupo la que nunca se había reportado evidencias macroscópicas de infección (FAC1). Además, se obtuvo la misma secuencia de CePV tanto de lesiones tipo "tattoo" como en muestras de piel sin evidencia clínica de la enfermedad, así como en ambas instalaciones y, además, mostró una alta homología con secuencias anteriores obtenidas de cetáceos salvajes procedentes del Atlántico Norte y Sur. Esto último plantea la cuestión de si este patógeno ha persistido en zoológicos y acuarios a través de generaciones desde la introducción de los individuos originales capturados en alrededor de los años 90, siendo capaz de producir infecciones latentes, y si la progresión de la

enfermedad puede depender de estímulos ambientales, de la carga viral o del buen estado de salud/inmunológico de los animales individuales.

5.2.3 ENFERMEDADES VÍRICAS DE LA PIEL EN CETÁCEOS ODONTOCETOS: CARACTERIZACIÓN MACROSCÓPICA, HISTOPATOLÓGICA Y MOLECULAR DE PATÓGENOS SELECCIONADOS

El CePV en cetáceos es ampliamente conocido por causar lesiones cutáneas bastante características conocidas como lesiones "tattoo", las cuales se tratan de lesiones puntiformes o anulares con un patrón punteado en el centro, de apariencia plana o ligeramente elevada, solitarias o coalescentes. Estas lesiones pueden desaparecer rápidamente para reaparecer de nuevo o persistir durante un largo periodo de tiempo. En la actualidad, y probablemente debido a su distinguible manifestación cutánea, no se ha reportado la detección de este patógeno en otro tipo de lesiones cutáneas. Tampoco se ha contemplado la posible consideración de la presencia de patógenos concomitantes en estas lesiones que pudieran influir en su desarrollo distintivo a lo largo del tiempo.

En relación con lo anterior, el presente trabajo consiste en un estudio retrospectivo en el que se establece la identificación y clasificación macroscópica de ocho categorías diferentes de lesiones de piel ("tattoo" (ovalado, coalescente y serpiginoso); lesiones negras, lesiones con bordeado blanco; pálidas; ulcerativas; en diana; en anillo; y tortuosas) con sus respectivas descripciones a partir de 55 lesiones cutáneas de 31 cetáceos varados en las costas canarias entre los años 2011 - 2021. Se realizaron análisis histopatológicos y moleculares con el objetivo de detectar no sólo CePV sino otros patógenos emergentes tales como herpesvirus (HV) y morbillivirus (CeMV). Entre los resultados obtenidos, lo más destacable fue que, molecularmente, 47 lesiones cutáneas resultaron positivas (85,45%) a uno o más de los agentes virales seleccionados en el presente estudio, y sólo ocho resultaron negativas (14,15%). Así mismo, se detectó la coinfección de CePV y HV en nueve lesiones de ocho cetáceos (16,36%), siendo este estudio el primero en reportar la comorbilidad de ambos patógenos en cetáceos. Además, se logró una correspondencia microscópica factible entre las lesiones positivas a CePV y HV, lo que permitió distinguir histológicamente qué patógeno se detectó en cada lesión, o incluso en qué lesión se detectaron ambos virus. Además, se logró la caracterización macroscópica e histológica de las lesiones positivas tipo "tattoo" y de las que presentaban tractos tortuosos.

5.2.4 CONCLUSIONES

1. Los cetáceos varados ofrecen la oportunidad de realizar estudios piloto como la replicación de nuevos protocolos de muestreo. Asimismo, se ha comprobado que los muestreadores celulares citológicos son una técnica fiable para muestrear lesiones cutáneas tipo tatuaje, obteniendo suficiente material genético para detectar CePV, presentando una eficacia similar o incluso mejor que las biopsias cutáneas dependiendo del método de extracción genómica utilizado.

2. Mediante el uso de muestreadores celulares citológicos, es posible detectar CePV de forma no invasiva reduciendo el impacto sobre el bienestar de los cetáceos bajo cuidado humano. Su practicidad, eficiencia de tiempo en la obtención de muestras e idoneidad para su uso tanto por veterinarios como por cuidadores hacen de este dispositivo una potencial alternativa de muestreo a las biopsias cutáneas.

3. Los muestreadores celulares citológicos logran, por primera vez en cetáceos, la detección de CePV tanto en lesiones cutáneas tipo tatuaje como en piel aparentemente sana de individuos bajo cuidado humano que no han mostrado previamente signos clínicos de enfermedad cutánea.

4. La detección en cetáceos bajo cuidado humano de dos zoológicos diferentes de la misma secuencia de CePV con alta homología a secuencias obtenidas previamente de cetáceos salvajes del Atlántico Norte y Sur puede significar que el virus podría haber sido transmitido entre individuos durante años desde la introducción de los primeros cetáceos salvajes en zoológicos y acuarios.

5. El estudio de una amplia gama de lesiones cutáneas y el análisis molecular de patógenos como el CePV, el HV y el CeMV ha permitido mejorar nuestro conocimiento de la patogénesis de estas enfermedades emergentes. Así, se ha podido detectar por primera vez la presencia de HV en lesiones que hasta ahora se asociaban exclusivamente a CePV y, además, se ha comprobado la alta prevalencia de estos agentes virales en lesiones cutáneas.

6. La caracterización macroscópica e histopatológica de las lesiones cutáneas junto con las detecciones moleculares obtenidas ha permitido diferenciar lesiones asociadas tanto a CePV como a HV específicamente, así como lesiones asociadas a ambos agentes. Por ejemplo, lesiones macroscópicas con un patrón tortuoso podrían asociarse al VH.

7. La excepcional detección en este estudio de la comorbilidad de CePV y HV en las mismas lesiones cutáneas plantea nuevos interrogantes sobre la interacción de estos patógenos y sus efectos en la infección y patogénesis de uno sobre el otro.

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