UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Università degli Studi di Padova

Dipartimento di Biomedicina Comparata e Alimentazione

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE VETERINARIE

INDIRIZZO COMUNE

CICLO XXVIII

THE IMMUNE SYSTEM OF CETACEANS AND THEIR INTERACTION WITH DOLPHIN MORBILLIVIRUS

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È una follia odiare tutte le rose perché una spina ti ha punto,

abbandonare tutti i sogni perché uno di loro non si è realizzato,

rinunciare a tutti i tentativi perché uno è fallito.

È una follia condannare tutte le amicizie perché una ti ha tradito,

non credere in nessun amore solo perché uno di loro è stato infedele,

buttare via tutte le possibilità di essere felici solo perché qualcosa non è andato per il verso giusto.

Ci sarà sempre un'altra opportunità, un'altra amicizia, un altro amore, una nuova forza.

Per ogni fine c'è un nuovo inizio.

Antoine de Saint-Exupéry, "Il Piccolo Principe"

ABSTRACT

Immunology of marine mammals is a relatively new field of scientific studies and its monitoring plays an important role on the individual and group management of these animals, as well as an increasing value of environmental health indicator: cetaceans are viewed as environmental sentinels. The current knowledge about the immune system of cetaceans and its function is recognized as incomplete.

Therefore this study aims to implement the knowledge on the immune response in normal conditions in cetaceans stranded along the Italian coastline in order to provide a base-line useful for assessing the immune status of bottlenose dolphin (*Tursiops truncatus*) and striped dolphin (*Stenella coeruleoalba*), the species most found in our seas and included in some international conventions such as species with high protection.

The selection of cetaceans to be included in the study was based on the availability of samples of spleen, thymus and lymph node tissue of the animals, on the way (in formalin or frozen) and the state of preservation; They have in fact been preferred tissues of animals whose sampling occurred within 48 hours of death in order to reduce as much as possible post-mortem alterations. Thereafter, animals were divided into groups on the basis of information obtained from signaling, such as species, sex, age, and environment of origin, on the outcome of virological investigations, microbiological, parasitological and toxicological, if performed, and the cause and/or the death mechanism.

On the lymph node slides obtained from the formalin-fix, paraffin-embedded samples it was made basic hematoxylin-eosin staining and immunohistochemical staining using the following antibodies: *Monoclonal Mouse Anti-Human CD3* to identify T lymphocytes, *Monoclonal Mouse Anti-Human CD20* for the identification of mature B lymphocytes and *Monoclonal Mouse Anti-Human HLA-DR Antigen, Alpha-Chain* for the identification of the major histocompatibility complex type II.

It is also arranged to validate by means of the Western blotting technique antibodies used in immunohistochemistry (IHC). The use of these antibodies was valid only for humans and some domestic animals (dogs and cats), but not in the species of interest to us such as bottlenose and striped dolphins.

With the antibodies mentioned above, also the lymphoid tissues of dolphins stranded along the coasts of the Canary Islands were tested and used as negative control since the death was probably due to collisions with boats/ships.

Finally, a semi quantitative samples analysis was performed by acquisition of slides via slide scanner for digital pathology (D-SIGHT) and a manual count of the number of cells positive for each antibody in 10 fields to 40x magnification, considered to be representative of the entire organ, and these data were statistically analyzed using the T TEST method. Statistical analysis showed no significant correlation between the variables considered and the expression of the different lymphocyte populations.

Future analysis should be aimed at analyzing the relationship between CD4+ and CD8+ cells in order to understand the effect of the major immunomodulatory pathogens, such as the dolphin morbillivirus, on sub-populations of T cells. It would also be important to compare the data obtained on the immune system with those obtained from the same samples as regards the search for environmental pollutants in order to understand the real role on the health of marine mammals present in our seas.

The study was not focused only on the immune response of the guests to the different pathogens, but we concentrate our work also on the most important pathogen currently causing die-offs of marine mammals: the dolphin morbillivirus (DMV). In particular, from tissues of a positive DMV fin whale (*Balaenoptera physalus*) stranded along the Italian coastline in October 2013 it was possible to completely sequence the P / V / C gene (1520 bp), M (1007 bp) , N (1573 bp), F (1659 bp) and H (1814 bp) respectively coding for the phosphoprotein and two virulence factors (V and C), for the matrix protein, the nucleoprotein, the fusion protein and the hemagglutinin protein. The complete sequences of the mentioned genes were deposited in GenBank (GenBank provisional Acc. No. KU977449, KU977450, KU977451, KU977452 and KU977453). The isolation of the virus in the tissues was made possible by molecular techniques such as RT-PCR using primers designed ad hoc and cloning using plasmid vector. The nucleotide changes, and consequently the amino acid variations, of each individual gene were subsequently analyzed and compared with the viral genome of the preceding epidemics. It was then possible to carry out a study of the tertiary structure of the viruses to see how these single mutations had a role in the structural change of the proteins themselves.

On the basis of this work we proceeded to identify an appropriate diagnostic technique usable even for large whales where correct sampling and appropriate samples storage is not always possible in field condition. We develop a nested RT-PCR which allows the unambiguous identification of a 200 bp fragment of the DMV genome corresponding to a highly conserved part of the gene H. This technique permits, if the viral genome is particularly fragmented because of the poor tissue conservation status, to verify the positivity to the DMV in tissue analysis, to isolate part of the

virus, otherwise difficult to be isolated, and it can be sequenced. This technique was later used successfully for identification and sequencing of DMV in the tissues of three sperm stranded in Vasto beach in 2014 (GenBank Acc. No. KU886570).

We can therefore say that the DMV is affecting species that until recently were considered species not susceptible to infection, such as fin whale and sperm whale. Individuals of these new species affected by the infection are mainly young animals or even pup and the virus isolation in a *fetus* organs confirms the possibility of vertical transmission.

Given the species barrier carried out by the virus, its point-like changes in the genomic sequence of the virus and incidence of positivity in 19% of the target species in 2015 we can claim to be in a situation where the virus is endemic in the Mediterranean Sea resulting increase in the infectious pressure.

Future studies will aim to understand more precisely the role of individual amino acid changes and their influence on the virulence and pathogenicity of the virus through the use of crystallography, to study the structure of the virus cellular receptor, CD150, to understand its real interaction with the virus, and to try to understand the real significance of the DMV in the ecology of the entire cetaceans population in the Mediterranean Sea.

RIASSUNTO

L'immunologia dei mammiferi marini è un campo relativamente recente degli studi scientifici e il suo monitoraggio ha un ruolo importante sulla gestione individuale e di gruppo di questi animali, nonché un crescente valore come indicatore della salute ambientale: i cetacei sono infatti considerati sentinelle ambientali. Le conoscenze attuali relative al sistema immunitario dei cetacei e alla sua funzione sono però incomplete.

Questo studio si prefigge dunque lo scopo di implementare le conoscenze sulla risposta immunitaria in condizioni di normalità nei cetacei spiaggiati lungo le coste italiane al fine di fornire una base-line utile per valutare lo stato immunitario di tursiope (*Tursiops truncatus*) e stenella (*Stenella coeruleoalba*), specie maggiormente presenti nei nostri mari ed incluse in alcune convenzioni internazionali come specie ad elevata protezione.

La selezione degli animali da inserire nello studio si è bastata sulla disponibilità di campioni di milza, timo e tessuto linfonodale dei vari soggetti, sulle modalità (in formalina o congelati) e sullo stato di conservazione degli stessi; sono stati infatti preferiti tessuti di animali il cui campionamento è avvenuto dell'arco delle 48 ore dal decesso al fine di ridurre il più possibile le alterazioni post-mortali. Successivamente gli animali sono stati divisi in gruppi sulla base delle informazioni ottenute dal segnalamento come specie, sesso, classe di età e ambiente di provenienza, sull'esito di indagini virologiche, microbiologiche, parassitologiche e tossicologiche, qualora eseguite, e la causa e/o meccanismo del decesso.

Sulle sezioni di linfonodo ottenute dai campioni in formalina è stata effettuata la colorazione di base ematossilina-eosina e le colorazioni immunoistochimiche usando gli anticorpi di seguito elencati: *Monoclonal Mouse Anti-Human CD3* per l'identificazione dei linfociti T, *Monoclonal Mouse Anti-Human CD20* per l'identificazione dei linfociti B maturi e *Monoclonal Mouse Anti-Human HLA-DR Antigen, Alpha-Chain* per l'identificazione del complesso maggiore di istocompatibilità di tipo II.

Si è inoltre provveduto a validare mediante la tecnica del Western Blotting gli anticorpi che precedentemente sono stati usati in immunoistochimica (IHC). L'uso di questi anticorpi era validato solo per l'uomo ed alcuni animali domestici (cane e gatto), ma non nelle specie di nostro interesse quali tursiope e stenella.

Sono stati inoltre testati tramite IHC, con gli anticorpi sopra citati, i tessuti linfoidi di cetacei spiaggiatesi lungo le coste delle isole Canarie usati come controllo negativo in quanto morti verosimilmente a causa di collisioni con barche/navi.
Infine è stata effettuata un'analisi semi quantitativa dei campioni mediate l'acquisizione dei vetrini tramite l'acquisitore D-SIGHT e una conta manuale del numero di cellule positive per ogni anticorpo in 10 campi ad ingrandimento 40x ritenuti rappresentativi di tutto l'organo e su questi dati è stata eseguita un'analisi statistica con il metodo del T TEST.
L'analisi statistica non ha mostrato alcuna correlazione significativa tra le numerose variabili prese in esame e l'espressione delle diverse popolazioni linfocitarie. Analisi future dovranno essere volte ad analizzare il rapporto tra CD4 e CD8 al fine di capire l'effetto dei principali patogeni immunodepressori, quali il dolphin morbillivirus, sulle sub-popolazioni di linfociti T. Sarebbe inoltre importante comparare i dati ottenuti sul sistema immunitario con quelli ottenuti dagli stessi campioni per quanto riguarda la ricerca di agenti inquinanti ambientali per capirne in reale ruolo sulla salute dei mammiferi marini presenti nei nostri mari.

La ricerca non si è però focalizzata solamente sulla risposta immunitaria degli ospiti ai vari patogeni, ma ci siamo concentrati sul patogeno più importante tra quelli che al momento causano morie tra i mammiferi marini: il dolphin morbillivirus (DMV). In particolare, a partire da tessuti di un esemplare DMV positivo di balenottera comune (*Balaenoptera physalus*) spiaggiatesi lungo le coste italiane nell'ottobre 2013 è stato possibile sequenziare completamente i geni P/V/C (1520 bp), M (1007 bp), N (1573 bp), F (1659 bp) ed H (1814 bp) codificanti rispettivamente per la fosfoproteina e due fattori di virulenza (V e C), per la proteina di matrice, la nucleoproteina, la proteina di fusione e l'emoagglutinina. Le sequenze complete dei geni sopracitati cono state depositate in GenBank (GenBank provisional Acc. No. KU977449, KU977450, KU977451, KU977452 e KU977453). L'isolamento del virus nei tessuti dell'animale è stato possibile grazie a tecniche biomolecolari quali RT-PCR con uso di primers disegnati ad hoc e clonaggio mediante vettore plasmidico. I cambiamenti nucleotidici, e di conseguenza amminoacidici, di ogni singolo gene sono stati successivamente analizzati e confrontati con il genoma virale delle precedenti epidemie. È stato poi possibile eseguire uno studio della struttura terziaria del virus per visualizzare come questi cambiamenti puntiformi avessero un ruolo nel cambiamento strutturale delle proteine stesse.
Sulla base di tale lavoro di sequenziamento abbiamo provveduto a individuare una tecnica diagnostica opportuna utilizzabile anche su animali di difficile gestione (difficoltà di campionamento e di conservazione opportuna dei campioni) approntando una nested RT-PCR che

permetta l'identificazione univoca di un frammento di genoma di 200 bp corrispondente ad una parte altamente conservata del gene H. Questa tecnica permette, qualora il genoma del virus sia particolarmente frammentato a causa del cattivo stato di conservazione dell'animale, di verificare la positività a DMV del tessuto in analisi, di isolare parte del virus, altrimenti difficilmente isolabile, e di poterlo sequenziare. Tale tecnica è stata poi utilizzata con successo per l'identificazione e il sequenziamento di DMV nei tessuti di 3 capodogli spiaggiatisi a Vasto nel 2014 (GenBank Acc. No. KU886570).

È possibile dunque affermare che il DMV sta colpendo specie che fino a poco tempo fa erano considerate specie non sensibili all'infezione, quali balenottera comune e capodoglio. Gli animali di queste nuove specie colpiti dall'infezione sono prevalentemente animali giovani o addirittura cuccioli e l'aver isolato il virus negli organi di un feto conferma la possibilità di trasmissione dello stesso per via verticale.

Visto il salto di specie effettuato dal virus, i suoi cambiamenti puntiformi nella sequenza genomica e un'incidenza di positività del virus nelle specie target del 19% nel 2015 possiamo affermare di essere in una situazione in cui il virus è endemico nel Mar Mediterraneo con conseguente aumento della pressione infettante.

Gli studi futuri in questo ambito saranno volti a capire in maniera più precisa il ruolo dei singoli cambiamenti aminoacidici e la loro influenza sulla virulenza e patogenicità del virus grazie all'impiego della cristallografia, a studiare la struttura del recettore cellulare del virus, il CD150, per capire la sua reale interazione con il virus, e a cercare di capire la reale rilevanza del DMV nell'ecologia dell'intera popolazione di balenottera comune nel mediterraneo.

CONTENTS

1. BACKGROUND

The increasing susceptibility to diseases in different Mediterranean whales' and dolphins' populations has led to speculation about a possible negative influence of multiple environmental factors on the immune system and therefore on the health status of marine mammals. Despite current efforts in studying the immunology of marine mammals, several aspects of immune functions in these species remain unknown.

Lymphoid organs of whales and dolphins are primarily affected by infectious agents and inflammatory changes (Beineke et al., 2010). Many cases of distemper-like diseases due to cetacean morbilliviruses, including the porpoise morbillivirus (PMV) and dolphin morbillivirus (DMV), have been commonly observed in different cetacean species, such as striped dolphins (*Stenella coeruleoalba*), bottlenose dolphins (*Tursiops truncatus*), common dolphins (*Delphinus delphi*) and harbor porpoises (*Phocoena phocoena*) (Van Bressem et al., 2014). Besides nervous dysfunctions as well as respiratory and gastrointestinal symptoms, morbillivirus infection leads to leukopenia and immunosuppression in aquatic mammals (Di Guardo et al., 2005; Jensen et al., 2002; Kennedy, 1998; Muller et al., 2004; Wohlsein et al., 2007). Accordingly, affected animals are prone to opportunistic infections, such as bacterial pneumonia, as well as parasitic and mycotic diseases (Domingo et al., 1992; Jensen et al., 1998). Similar to distemper in carnivores, cetacean morbillivirus disease induce an extensive lymphocytolysis and nuclear inclusion bodies in remaining lymphoid cells associated with a generalized depletion of lymphoid organs (Beineke et al., 2009). Multinucleated giant cell or syncytial cell formation of lymphoid cells are a frequent feature in PMV- and DMV-infected cetaceans (Di Guardo et al., 2005; Kennedy, 1998).

Furthermore, a progressively expanding DMV host range is highlighted by recently documented cases in Mediterranean fin whales (Di Guardo et al., 2011; Mazzariol et al., 2012, 2016; Casalone et al., 2014), in sperm whales (Centelleghe et al., 2016) and, even more strikingly, also in an under human care common seal (*Phoca vitulina*) (Mazzariol et al., 2013).

The recent discoveries of several new morbilli-related viruses in bats (Drexler et al., 2012), as well as a new potentially feline morbillivirus associated with tubulointerstitial nephritis in domestic cats (Woo et al., 2012) representing a basal divergence in the genus, are likely to lead to understanding the evolution of morbilliviruses.

Morvilliviruses are a growing concerning viral group because of their ability to infect multiple species, often endangered, as recently reported for canine distemper virus (CDV) infected Amour

tiger (*Panthera tigris altaica*) and lion (*Panthera leo*) populations (Seimon et al., 2013; Viana et al., 2015) probably caused by a spill-over of CDV from domestic dog associated with severe decline in wild carnivores worldwide and mass dog vaccination.

On the basis of what above stated, the present study is focused on understanding marine mammals immune system characterization as well as on DMV, one of the main pathogens affecting the immune system itself. In fact, adequate research efforts would be needed to understand the interaction between cetaceans immune system, the ecosystem and the DMV tropism in relation to the different cetacean species and to their susceptibility to infection

1.1 MARINE MAMMALS IMMUNE SYSTEM

The lymphatic system of mammals includes the lymph nodes, precisely structured masses of lympho-reticular tissue occurring at intervals along the lymphatic vessels; furthermore, diffuse and organized masses of lymphocytes are associated with mucosal surfaces, including tonsils; the spleen and the thymus.

For many terrestrial species, the immune system has been studied in great detail and therefore its development is known to vary among mammals. In some species, such as ruminants, the system is fully developed at birth, while in rodents, for example, full development seems to require exposure to environmental antigens (Banks, 1982). Some lymphoid organs, such as the spleen, appear to be active throughout life, while others, such as the thymus, involute relatively early in life (Burkitt et al. 1993).

For most cetaceans' species, reports found in the literature about the development and structure of the lymphoid system are scanty, fragmented, and frequently old. Notable exceptions include an extensive microscopic examination on the lymphoid organs in belugas (*Delphinapterus leucas*) and in bottlenose dolphins detailing the morphological architecture of the immune system (Romano et al., 1993; Cowan and Smith, 1999). Another previous study also examined lymphoid organs in selected marine mammal species, providing general histological information (Simpson and Gardner, 1972). However, no other comprehensive studies have been performed and published on this system in cetacean species except for an evaluation of cellular and humoral immune responses in beluga whale (*Delphinapterus leucas*) and harbor porpoise (*Phocoena phocoena*) and the immunophenotyping of their lymphoid cells (Beineke et al., 2010).

1.1.1 Lymph nodes

Lymph nodes can be found grouped associated to specific systems and organs and in well defined anatomical regions.

A striking feature of the lymph node groups of cetaceans is the variability of definition of the individual nodes. In some animals nodes within a group may be closely applied to each other, but remain distinct, while in other animals the nodes fuse to form an irregular, lobular mass (Beineke et al., 2010).

The cervical and pelvic nodes have a relatively simple architecture, while the visceral nodes all contain variable amounts of smooth muscle in the capsule and the trabeculae, depending on their location. The mesenteric nodes are the most muscular of all the node groups. This muscle occurs as a component of the thick capsule and effectively encapsulates the nodes and in addition to

extending along the trabeculae, forms an interlacing network throughout the node (Cowan and Smith, 1999). The other visceral nodes all have lesser amounts of smooth muscle in the capsules and trabeculae. For example, the nodes of the respiratory tract all have thick capsules, with small amounts of smooth muscle. The most distinctive feature of these nodes is the antler-like branching of the blunt collagenous trabeculae, a pattern which occurs within a short distance of the capsule. The clear implication is that the visceral nodes are contractile organs, having an important, active role in moving as well as filtering lymph (Cowan and Smith, 1999).

Both somatic and visceral node groups have the general structure of sinuses and cords, and in responding to antigenic challenge will produce prominent follicles with germinal centres, even if they are frequently absent (Romano et al., 1993). Marginal sinuses are incomplete. Germinal centres, if present, are frequently found deep in the nodes, and some have likened this arrangement to an inverted architecture, as described in pigs (Moskov et al. 1969).

1.1.2 Thymus

The thymus of dolphins presents all the typical features of the mammalian thymus, with cortex, medulla, Hassall's corpuscles and epithelial reticulum. In the youngest animals, it extends from the arch of the aorta, where it invests the brachiocephalic vessels and partly or completely overlies the thyroid gland. Because of its color, soft texture, and lobular architecture, the thymus is easily mistaken for adipose tissue.

The cetacean thymus follows the microscopically typical mammalian plan with a cortex, medulla, and Hassall's corpuscles (Cave, 1980; Romano et al. 1993; Cowan, 1994). An epithelial reticulum has been demonstrated using a labelled monoclonal antibody against cytokeratin (Cowan, 1994).

Since the thymus can be observed macroscopically in health adult individuals and even remnants in senile harbor porpoises and bottlenose dolphins, thymic involution is regarded as a slow progressive age-related process in these cetaceans (Cowan, 1994; Wunschmann et al., 1999). It is clear that lymphocyte depletion occurs over time, but the age or rate of progression at which this occurs is not determined for any cetacean species (Beineke et al., 2010).

1.1.3 Spleen

The spleen of dolphins is a slightly flattened globe, most often greyish-blue, but occasionally covered in part with whitish patches, representing fibrous thickening of the capsule (Beineke et al., 2010).

The general architecture of the spleen is similar among different cetacean species. The capsule is double-layered, with a fibrous outer layer, and a fibromuscular inner layer. Depending on its state of reactivity, the cut surface of the spleen may display white, pinpoint nodules easily visible, which represent activated germinal centres (follicles) formed in peri-arterial lymphoid sheaths (Cowan and Smith, 1999).

The relative size of the cetacean spleen is small compared with land mammals (Bryden, 1972), approximating 0.2%of the animal's total body weight (Slijper, 1958). Some studies observed that, as in most mammals, the cetacean spleen reaches maximum size with the onset of puberty, and subsequently decreases in relative and absolute weight with increased age (Bryden, 1972). In general, the cetacean spleen is a single organ, but accessory spleens are common, found in 21% of common dolphin and 18% of striped dolphins (de Olivera e Silva, 2014).

As in land animals, the cetacean spleen is composed of white pulp, consisting of lymphoid nodules developed at arterial terminals, evenly distributed throughout the red pulp. Peri-arterial lymphatic sheaths are characteristically prominent. Lymphoid nodules are composed of small to medium-sized lymphocytes (Romano et al. 1993). Germinal centres may be identified as white granules on gross inspection, but as they reflect a reactive state, they are inconstant and often absent (Nakamine et al. 1992). In many cetacean species, the splenic capsule has 2 layers, an outer fibrous and an inner muscular layer. Trabeculae extend from the capsule into the parenchyma, each bearing arteries and veins. (Cave, 1980).

1.1.4 Immunophenotyping of cetacean lymphoid cells

Human cell surface antigens have been cataloged in international workshops (cluster of differentiation [CD]) and markers for detecting CD-homologues have been described for several animal species. However, only few reports mention the applicability of specific leukocyte markers in cetaceans (Beineke et al., 2010).

The specificity of cross-reacting bovine, human, ovine and murine monoclonal antibodies directed against different leukocyte subsets and the major histocompatibility complex class (MHC) II antigen of peripheral blood lymphocytes of beluga whales and bottlenose dolphins have been confirmed by immunoprecipitation and flow cytometry (De Guise et al., 1997; Romano et al., 1992; Shirai et al., 1998). Furthermore, cross-reacting markers directed against various cell surface antigens of the hematopoietic system, including T cell, B cell, histiocytic and MHC II antigens have been established for common dolphin, striped dolphin, bottlenose dolphin and harbor porpoise lymphoid tissues using immunohistochemistry (Beineke et al., 2001; Zabka and Romano, 2003). Similarly, histiocytic cells, particularly resident and inflammatory macrophages can be detected by

cross-reacting human antibodies directed against the macrophage-associated antigens CD163, CD204 and lysozyme in short-finned pilot whales (*Globicephala macrorhynchus*) and Risso's dolphins (*Grampus griseus*) using immunohistochemistry (Kawashima et al., 2004; Komohara et al., 2006). Bottlenose dolphin specific monoclonal antibodies for the detection of CD2, CD19, CD21 and CD45R antigens as well as the adhesion molecule b-2-integrine have been produced and characterized by flow cytometry and immunoprecipitation (De Guise et al., 2002 and 2004). Furthermore, T cells are recognized by the CD2 marker, while B lymphocytes are predominantly labeled by monoclonal anti-CD19 and -CD21 specific antibodies, using immunohistochemistry (De Guise et al., 2002). Besides, B cells and a subset of T cells are labeled by the CD45R marker (De Guise et al., 1998).

1.1.5 Effect of environmental contaminants on the cetaceans immune system

The high trophic feeding level of top predator cetaceans predisposes them to the bioaccumulation of persistent chemical compounds. Numerous studies reported the growing evidence for a potential negative impact of environmental contaminants on the immune system and subsequently on the health status of marine mammals (Siebert et al., 1999; Jepson et al., 2016).

Despite these hypotheses, a real effect of xenobiotics on the immune system, especially the dioxin-like PCBs has been established in laboratory rodents (Ross et al., 1997). However, only few studies focused on the influence of xenobiotics on the immune function of whales and dolphins.

A reduced mitogen-induced T cell proliferation associated with elevated PCB and p,p0-dichlorodiphenyltrichloethene (DDT) blood levels has been determined in free-ranging bottlenose dolphins on the coast of Florida, suggestive of a contaminant-induced inhibition of the cellular immune response. However, definitive conclusions concerning their impact on the health status are limited due to the small number of investigated dolphins and lack of controls animals (Lahvis et al., 1995). In other field studies of harbor porpoise populations, thymic atrophy and splenic depletion were significantly correlated to elevated body burdens of polybrominated diphenyl ether (PBDE) and PCBs (Beineke at al., 2005), but remained undetermined if changes in thymus and spleen are primarily contaminant-induced or a sequel of infectious disease, exhaustion and cachexia in most stranded harbor porpoises.

However, lipolysis and mobilization of stored lipophilic compounds during physiological (i.e. pregnancy and milking) and pathological (i.e. emaciation) condition might lead to the observed elevated blood levels of xenobiotics in diseased harbor porpoises. In addition, lymphoid depletion is primarily associated with elevated PBDE levels, while there is no correlation with the health status and nutritional state in by caught animals, supporting the hypothesis of a contaminant-induced

immune deficiency (Beineke et al., 2005). The immunotoxic effect of several xenobiotics on cetacean blood leukocytes at concentrations equivalent to those observed in wildlife marine mammal populations has been verified in vitro.

Additional toxicants, such as methylmercury and heavy metals are also suspected to negatively influence the immune system and increase disease susceptibility of harbor cetaceans (Siebert et al., 1999; Jepson et al., 2016) and in sperm whales (Mazzariol et al., 2011)

In vitro experiments confirmed the negative influence of heavy metals on bottlenose dolphin leukocytes. Particularly mercury and cadmium decrease cell viability, phagocytosis and proliferation of leukocytes. In addition, they trigger lymphocyte apoptosis in concentrations equivalent to those reported in free-ranging cetaceans (Camara Pellisso et al., 2008). Similarly, in vitro exposure to mercury chloride and cadmium chloride decreases beluga whale splenocyte and thymocyte proliferation. Furthermore, mercury compounds induce cell death of mitogen-stimulated beluga whale thymocytes (De Guise et al., 1996).

1.1.6 Effect of diseases on the cetaceans immune system

Lymphoid organs of whales and dolphins are primarily affected by infectious agents and inflammatory diseases. Epidemics and sporadic cases of distemper-like diseases due to cetacean morbilliviruses, including the porpoise morbillivirus (PMV) and dolphin morbillivirus (DMV),
have been observed in different cetacean species, such as striped dolphins, bottlenose dolphins, common dolphins, harbor porpoises and white-beaked dolphins (*Lagenorhynchus albirostris*). Besides nervous dysfunctions as well as respiratory and gastrointestinal symptoms, morbillivirus infection leads to leukopenia and immunosuppression in aquatic mammals (Di Guardo et al., 2005; Wohlsein et al., 2007). Accordingly, affected animals are prone to opportunistic infections, such as bacterial pulmonary infections as well as parasitic and mycotic diseases of the CNS (Domingo et al., 1992). Similar to distemper in carnivores, cetacean morbillivirus infections induce an extensive lymphocytolysis with acidophilic cytoplasmic and nuclear inclusion bodies in remaining lymphoid cells associated with a generalized depletion of lymphoid organs (Beineke et al., 2010).

1.2 DOLPHIN MORBILLIVIRUS

Cetacean morbillivirus (CeMV) is a recently described member of the genus *Morbillivirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, Order Mononegavirales. Other important pathogens in the genus *Morbillivirus* are measles virus (MV) in humans and other primates, rinderpest (RV) and peste des petits ruminants viruses (PPRV) in artiodactyls, canine and phocine distemper viruses (CDV and PDV) in carnivores and tentatively, a paramyxovirus from domestic cats currently named feline morbillivirus (Barret, 1999; Hall, 1995; Woo et al., 2012). CeMV includes three well characterized strains: the porpoise morbillivirus (PMV), first isolated from harbor porpoises (*Phocoena phocoena*) from Northern Ireland (McCullough et al., 1991), the dolphin morbillivirus (DMV), first isolated from Mediterranean striped dolphins (*Stenella coeruleoalba*) (Domingo et al., 1990) and the pilot whale morbillivirus (PWMV), recovered from a long-finned pilot whale (*Globicephala melas*) stranded in New Jersey, USA (Taubenberger et al., 2000).

1.2.1 Molecular characteristics and cellular receptors

Morbilliviruses are unsegmented, linear negative-sense, single-stranded RNA viruses. The DMV genome is 15,702 nucleotides long and consists of six transcription units that encode six structural proteins, the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the fusion glycoprotein (F), the haemagglutinin glycoprotein (H) and the RNA-dependent RNA polymerase (L), as well as two virulence factor proteins (C and V) (Barret et al., 1993; Rima et al., 2005).

RNA viruses are characterized by an extremely high mutation rate (i.e.~10-2-10-5 mutations/site/replication) which makes them extremely prone to genotypic and phenotypic changes that can lead to the emergence of variants with different immunological properties, virulence or host tropism (Duffy et al., 2008).

PMV and DMV are antigenically more closely related to the ruminant morbilliviruses and MV than to the distemper viruses (Osterhaus et al., 1995). Sequencing of the P, N, F and M genes further demonstrated and confirmed that PMV and DMV are closely related and that they form a separate group within the *Morbillivirus* genus, closer to the ruminant viruses and measles virus (MV) than to the CDV/PDV group (Bolt et al., 1994; Banyard et al., 2008 and 2011). The close genetic relationship between cetacean and ruminant morbilliviruses has led to the suggestion that they may have a common ancestor (Barret et al., 1993, Van Bressem et al., 2014): cetaceans belong indeed to the clade Cetartiodactyla. As several species of this clade are susceptible to RPV and PPRV (Kumar et al., 2014), it is possible that a host jump occurred between a cetacean and another member of the

Cetartiodactyla, and that ecological isolation led to distinct virus species (Van Bressem et al., 2014). The presence of similar host proteins and cell receptors in cetaceans and artiodactyls may favor cross-species transmission (Ohishi et al., 2010; Shimizu et al., 2013). However, further studies are needed to confirm this hypothesis.

The H glycoprotein is responsible for virus attachment to the host cell membrane and for cellular entry. The F glycoprotein causes fusion with the host cell membrane and, together with the M protein, invokes cell-to-cell fusion (Wild et al., 1991). H and F proteins interact with cellular receptors that allow virus entry and determine host susceptibility, tissue tropism and viral pathogenesis (Melia et al., 2014).

The signaling lymphocyte activation molecule (SLAM or CD150) and the poliovirus like receptor 4 (or nectin 4) have both been recently identified as the major receptors for wild-type morbilliviruses in immune and polarized epithelial cells, respectively (Ohishi et al., 2010; Shimizu et a., 2013; Melia et al., 2014). Most morbilliviruses, including MV, CDV, PDV, PPRV, and RPV use the SLAM of their respective host species as a receptor (Tatsuo et al., 2001).

The SLAM receptors have immunoglobulin-like variable (V) and constant-2 (C2) domains in their extracellular regions with the V domain providing an interface for the morbillivirus H glycoprotein. Substitution in the amino acid residues of this interface may lead to a loss of, a reduction in, or an increase in, viral infectivity. The morbillivirus H glycoprotein displays a strong affinity for this domain in its respective host (Shimizu et al., 2013). However, a recent study showed that only one amino acid change in H was required for functional adaptation of CDV to the human SLAM cell receptor *in vitro* (Bieringer et al., 2013).

Three residue substitutions (G68, H90 and H130) that introduced charge alteration and possible change in viral affinity were observed in the SLAM of the Delphinidae, while these residues were mostly conserved in the receptor of the other cetacean families. As morbillivirus mass mortalities have mostly been detected in the Delphinidae, it is possible that their SLAMs have a higher affinity for CeMV resulting in increased viral infectivity and dissemination (Shimizu et al., 2013).

1.2.2 Pathology and pathogenesis

Most morbilliviruses are lymphotropic and epitheliotropic. After initial replication in the lymphoid tissues, the virus is disseminated by infected lymphocytes through the lymphatic system and spreads to epithelial cells (Ludlow et al., 2015).

Acutely fatal CeMV infection is generally associated with severe multifocal to diffuse interstitial broncho-pneumonia characterized by necrosis of type I pneumocytes and bronchiolar epithelial cells, interstitial edema, type II pneumocyte hyperplasia, and formation of large syncytia in the

alveolar and bronchiolar lumina. Intracytoplasmic and intranuclear inclusion bodies can be noted and are sometimes numerous in respiratory epithelia, bronchiolar gland epithelia and the syncytial cells. Generalized lymphoid depletion with germinal center necrosis is usually present and syncytial cells (Warthin-Finkeldeytype) are often prominent in lymphoid tissues. There may be evidence of viral replication (inclusion bodies) in epithelia and neural cells of other body systems. Multifocal non-suppurative encephalitis may also be present (Kennedy et al., 1991; Domingo et al., 1992; Di Guardo et al., 1995).

Animals that survive the acute stage of infection may succumb to opportunistic infections (*Toxoplasma gondii*, herpesviruses, bacteria such as *Photobacterium damselae*, and fungi) as a consequence of the profound immunosuppression. This typical pattern has been commonly seen in odontocetes that died during worldwide outbreaks of CeMV (Di Guardo et al., 1995; Groch et al., 2014; Stephens et al., 2014; Di Guardo et al., 2013; Stone et al., 2011; Fernandez et al., 2008; Mazzariol et al., 2012; Soto et al., 2012). While some of the lesions typical of acute infection may no longer be present or be largely obscured by the inflammatory response to the opportunistic pathogens, non-suppurative demyelinating meningoencephalitis, often focally distributed, is a feature of sub-acute infection (Domingo et al., 1992).

Animals may survive the acute and sub-acute manifestations of infection but succumb sometime later to the secondary infections acquired as a result of viral immunosuppression, or from complications of CNS infection. Typically these animals are in poor body condition at the time of death and the proximate cause of death may be multifactorial. Invariably there are no or few lesions directly attributable to CeMV but viral antigen may be detectable by IHC in some lymph nodes and lungs and viral RNA may be amplified by RT-PCR (Lipscomb et al., 1994).

If the pathogenesis of CeMV is similar to that of MV, cetaceans that survived acute and sub-acute infection could show prolonged RNA persistence in the blood and lymphoid organs and could be molecularly positive in the absence of typical morbillivirus lesions (Lin et al., 2012).

Cetaceans that have cleared and resolved DMV systemic infection may develop a CNS form that is characterized by the presence of lesions and virus only in the brain (Di Guardo et al., 2013; Di Guardo and Mazzariol, 2016). This CNS form was consistently observed in striped dolphin (*Stenella coeruleoalba*) after the two epidemics in the Mediterranean sea (Soto et al., 2011). By contrast with the sub-acute cerebral CeMV infection, cytoplasmic or nuclear eosinophilic inclusions were only occasionally detected and syncytial cells were not observed in the CNS form. Many neuronal processes showed immunostaining for CeMV, and some areas had massive accumulation of CeMV-antigen, while contiguous zones of the brain had almost no staining. This suggests that the presence of CeMV was more the result of cell-to-cell spreading of infection rather than of a

multifocal infection indicative of blood-borne infection. The CNS form appears to share histological characteristics with subacute sclerosing panencephalitis (SSPE) and old dog encephalitis (ODE), chronic latent localized infections that affect humans and dogs, respectively, and are caused by defective forms of MV and CDV (Headley et al., 2009; Di Guardo and Mazzariol, 2016). In the three conditions perivascular cuffing, diffuse gliosis, and glial nodules with neurophagia were the most prominent changes (Domingo et al., 1995). Antigen and viral RNA could be detected in dolphin brains but the virus proved difficult to isolate. The mechanism for this is unknown but RT-PCR studies on the brain of striped dolphin chronically affected by CeMV suggest that the sequence of the P gene is different in these case (Soto et al., 2011). The role of cell receptors in the pathogenesis of this form of the disease should be further examined (Di Guardo, 2012).

It is possible that CNS persistence plays a role in the maintenance of strains in a sea basin, although, as a dead end infection, it is unlikely to contribute to virus transmission to other cetaceans (Van Bressem et al., 2014).

1.2.3 Viral transmission

Morbillivirus horizontal transmission is thought to occur mostly after the inhalation of aerosolized virus shed by infected individuals. This transmission is likely to be favored by a gregarious behavior of some species and a high density of cetaceans (Van Bressem et al., 1999). Transmission by inhalation of expired blowhole droplets possibly occurs during breathing in a synchronized fashion when large numbers of cetaceans are travelling and feeding together or are engaged in social activities (Van Bressem et al., 1999).

The first evidence that vertical transmission may occur was the detection of morbilliviral RNA in brain, lung, spleen, lymph node, and liver from the seven-month fetus of a DMV-infected long-finned pilot whale (*Globicephala melas*) stranded in the Balearic Islands in 2007 (Fernandez et al., 2008). These data suggest that CeMV infected females may transmit the infection to their fetuses and neonates *in utero* and probably during lactation (Van Bressem et al, 2014).

1.2.4 Epidemiology

Morbilliviruses are extremely infectious and are likely to infect most of the immunologically naive individuals in a population. These viruses require large populations of susceptible individuals to persist endemically, as there is no carrier state and infection confers lifelong immunity (Black, 1991).

However, the persistence of morbilliviruses in relatively small (possibly multispecies) host metapopulations remains an important unsolved problem in disease ecology (Almberg et al., 2010). Newborn individuals typically have maternal immunity if their mothers had previously been infected. After some months, this immunity is lost and the young individuals are fully susceptible to infection (Dobson et al., 1991).

CeMV infection has been detected using various techniques in several species of odontocetes and mysticetes worldwide (Table 1.1). In the absence of, or decrease in, herd immunity, outbreaks of lethal disease may occur in susceptible species, as has repeatedly been observed in Europe, the Americas, and Australia since the late 1980s (Van Bressem et al., 2014).

In the Mediterranean Sea, DMV caused two well-documented outbreaks of mass mortality in striped dolphin in 1990–1992 and in 2006–2008. The first outbreak started in Spain in 1990 and extended to France, Italy, Greece and Morocco, ending in 1992. Although precise mortality rates could not be determined, thousands of animals are thought to have died, most of these were adults (Aguilar et al., 1993). Serological surveys carried out during and after the epidemic indicated that in 1997–1999 only adult dolphins had DMV antibodies and that the prevalence of seropositivity in a small number of mature dolphins had decreased from 100% (N= 8) in 1990–1992 to 50% (N= 6) in 1997–1999. This suggested that the virus had not persisted in the animals after the epidemic ended, presumably because their abundance in the western Mediterranean Sea was too low to support endemic infection (Van Bressem et al., 2001). Histological and IHC surveillance further supported this hypothesis.

Between the end of 2006 and the beginning of 2007, at least 27 morbillivirus-infected long-finned pilot whales stranded along the southern Spanish Mediterranean coast and the Balearic Islands (Raga et al., 2008). In early July 2007 DMV-infected were observed in the Gulf of Valencia (Raga et al., 2008). The number of striped dolphins washed ashore from July through August 2007 in the Gulf was similar to that recorded in 1990 during the same months. The outbreak extended to France and Italy during the following months, also affecting mostly juveniles bottlenose dolphins likely because adults were still protected by immunity acquired during the 1990–1992 epidemic (Di Guardo et al., 2013; Raga et al., 2008). The virus strains amplified by RT-PCR were similar to those isolated during the 1990–1992 epidemic but not identical (Van Bressem, 2014). As well as the deaths caused by the acute infection, there were also several cases, ultimately lethal, of a chronic CNS form of infection in 1991–1994 and 2008–2011 in the western Mediterranean and in 2009–2011 in the Eastern Mediterranean (Soto et al., 2011; Di Guardo et al., 2013). In the Western Mediterranean chronic morbillivirus, encephalitis represented the most common single cause of stranding and death in mature striped dolphin in the years following a DMV epizootic (Soto et al.,

2011). Little is known about the impacts of the outbreak on populations of the other cetacean species affected (Van Bressem et al., 2014).

As both the 1990–1992 and 2006–2007 DMV epidemics started close to, or in, the Gibraltar Strait, it was suggested that DMV endemically infected cetaceans, possibly long-finned pilot whales transmitted the infection to striped dolphins with which they occasionally form mixed groups (Van Bressem, 2014). The finding of systemic morbillivirus infection in two adult striped dolphins stranded on the southwestern (Atlantic) coast of Spain, close to Gibraltar in 2011 and 2012 further indicates that this Strait plays an important role in the epidemiology of CeMV (Van Bressem, 2014). Environmental factors such as higher sea-surface temperatures and limited prey availability, as well as fisheries interactions, inbreeding, migration, and high contaminant loads may synergistically interact to increase the severity of the disease and favor transmission between species (Anguilar et al., 1994; Fossi et al., 2007). When CeMV herd immunity significantly decreases in Mediterranean cetaceans population, the animals will again be at risk for an epidemic (Van Bressem et al., 2014).

Recently, other 2 outbreaks have been reported along the Italian coastline: DMV RNA was detected by RT-PCR in brain and lung samples from 22 of 52 striped dolphins, in one of three bottlenose dolphin and one new-born fin whales (*Balaenoptera physalus)* stranded along the Italian coastline during an unusual mortality event in early 2013. However, none of the positive individuals had characteristic morbillivirus lesions and other infectious agents were concurrently detected in a high percentage of these individuals (Casalone et al., 2014; Mazzariol et al. 2015).

1.2.5 Diagnosis

Though virus isolation remains the gold standard for definitive diagnosis, it is challenging when dealing with stranded cetacean carcasses because of the poor conservation status. RT-PCR followed by sequencing has proven very helpful for obtaining rapid confirmation of CeMV infection, to differentiate between PMV and DMV and to identify new strains (Barret et al., 1993; Banyard et al., 2008; Grant et al., 2009). Histology and immunohistochemistry (IHC) have provided further confirmation of the disease and insights into its pathogenesis and have permitted differentiation between systemic disease and localized chronic infection of the central nervous system(CNS) (Soto et al., 2011; Di Guardo et al. 1995). Serological studies have also been useful for studying CeMV epidemiology, to assess the immune status of populations before and after an outbreak and to predict the occurrence of new epidemics (Van Bressem et al., 2014).

- Histology and Immunohistochemistry

Histology and IHC techniques should always be used to confirm the molecular diagnosis of systemic morbillivirus infection during an outbreak of mortality. Classical histological techniques have been used to investigate CeMV disease and pathogenesis since the first harbor porpoise and Mediterranean striped dolphin were suspected of dying of morbillivirus infection (Domingo et al., 1990; Kennedy et al., 1988). Immunohistochemistry has greatly enhanced the sensitivity and specificity of histopathological diagnosis by enabling the detection of morbillivirus antigen in cases where tissue preservation is poor or where classical lesions have been obscured by opportunistic pathogens. IHC studies have been conducted by using a commercially available monoclonal antibody against CDV N protein (Sierra et al., 2014; Di Guardo et al., 2013), a monoclonal antibody for PDV hemagglutinin (Domingo et al., 1990; Lipscomb et al., 1994), or a rabbit polyclonal antiserum to rinderpest virus (Yang et al., 2006). Specific monoclonal antibody against DMV or PMV proteins are not commercially available although they would be useful for accurate diagnosis and research in the future.

- Serology

Virus neutralization (VN) tests, plaque reduction (PR) assays and indirect enzyme-linked immunosorbent assays (iELISAs) are the main platforms used to detect antibodies against CeMV. The iELISA allows the detection of antibodies directed against the N, P, F and H CeMV proteins whereas only antibodies to the surface glycoproteins (H and F) are detected by the VN and PR assays. Morbilliviruses are antigenically closely related and may cross-neutralize one another. Thus, when working with cetaceans it is very important to use CeMV strains in the serological tests to avoid false negatives (Barret et al., 1993).

Indirect ELISAs were developed to analyze hemolyzed serum samples that could be cytotoxic and, as such, could prevent the detection of morbillivirus antibodies at low dilutions in virus neutralization tests(Van Bressem et al., 1998). The iELISA appears to be more sensitive than the classical VN test and may be useful as a serological tool for the mass screening of morbillivirus antibodies in cetaceans. A competitive ELISA using monoclonal antibodies against CDV and PDV was developed for testing sera from various species of marine mammals. Its main advantage over iELISAs is that a single anti-mouse immunoglobulin conjugate can be used on serum from any animal species (Saliki and Lehenbauer, 2001). However, sensitivity was lower for detection of cetacean compared to carnivore morbilliviruses.

The VN test is highly sensitive and very specific and is considered the most reliable assay for the detection of CeMV antibodies (Saliki and Lehenbauer, 2001). Antibody titers are expressed as the reciprocal of the highest dilution of sera that completely neutralizes cytopathic effects. Titers of

1:16 or higher are considered to be indicative of exposure to CeMV, although higher thresholds can be used to reduce the likelihood of false positives (Van Bressem et al., 2014).

- Reverse Transcription Polymerase Chain Reaction

A "universal" morbillivirus primer set (Barret et al., 1993), based on highly conserved regions of the morbillivirus P gene has been successfully used in frozen samples to detect CeMV by reverse transcription polymerase chain reaction (RT-PCR) during outbreaks worldwide (Van Bressem et al., 2014).

Using a similar approach, a different research group designed a protocol that allows amplification of degraded RNA in formalin-fixed paraffin embedded samples and in unfixed autolyzed tissues (Krafft et al., 1995). Since then, other primers including sets of "universal" morbillivirus primers based on the conserved N terminus of the morbillivirus N gene, were also successfully used to detect CeMV (Raga et al., 2008).

A real-time RT-PCR (rtRT-PCR) that targets the hypervariable C terminal domain of the N gene was developed for a rapid and differential detection of DMV and PMV. This test is rapid, very sensitive and specific for either DMV or PMV and does not cross-react with CDV, PDV, RPV, PPRV and MV. A rtRT-PCR assay that targeted the glyceraldehyde3-phosphate dehydrogenase (GAPDH) gene, as a house-keeping gene, was developed to determine whether total RNA extracted from stranded cetacean tissues is amplifiable (Gant et al., 2009). This test allowed for the detection of GAPDH gene sequences from 14 marine mammal species and is essential for interpreting negative results with the morbillivirus RT-PCRs. Another rtRT-PCR was later designed to amplify a highly conserved region within the F gene and to differentiate between DMV, PMV, and PWMV (Rubio Guerri et al., 2013).

More recently, a pan-marine mammal morbillivirus semi-nested RT-PCR using a degenerate set of primers targeting conserved sequences of the P gene was described for the detection of both pinniped and cetacean morbilliviruses (Sierra et al., 2014).

Clearly, with all the advances in molecular biology, diagnosing CeMV infection has become much faster, easier and more reliable. RT-PCR assays should be used together with the other techniques to distinguish among acute infection, prolonged persistence of morbillivirus RNA following CeMV acute disease, and chronic infection. When CeMV infection is detected in a novel host species, samples should be sequenced for species confirmation and identification and also sent to morbillivirus reference centers for genetic confirmation of the species involved (Van Bressem et al., 2014).

Significant progress in our understanding of the epidemiology, molecular biology and pathogenesis of CeMV have been made since PMV and DMV were first detected in small odontocetes in European waters in 1988–1992 (Van Bressem et al., 2014). Several techniques have been developed to optimize the diagnosis of CeMV infection, to differentiate the strains and to reduce the possibility of cross-contamination (Barret et al., 1993; Banyard et al., 2008; Gran et al., 2009). Serological assessment may enable prediction of future outbreaks. The development of Next Generation Sequencing technologies has greatly enhanced the detection and genetic characterization across all forms of life (Van Bressem et al., 2014).

Therefore, standard sampling and preservation protocols should be used during suspected morbillivirus outbreacks and complete genomes of CeMV strains should be sequenced (Van Bressem et al., 2014).

Table 1.1. CeMV infection in odontocetes and mysticetes CeMV infection in odontocetes and mysticetes in Mediterranean sea. Modify from Van Bressem et al., 2014.*

Species	Years	Countries	Epidemiological status	Diagnosis	Virus	Literature cited
S. coeruleoalba	1990-1992	Spain, France, Italy, Greece	Epidemic	VI, IHC,S, RT-PCR	DMV	Domingo et al., 1990; Di Guardo et al., 1995; Aguilar et al. 1993
S. coeruleoalba	2006-2008	Spain, France, Italy	Epidemic	IHC, RT-PCR	DMV	Di Guardo et al., 2013; Raga et al., 2008
T. truncatus	1994; 2007-2008; 2011	Israel, Spain, France, Italy,	Periodic mortalities	IHC, RT-PCR, S	DMV	Van Bressem et al., 2001; Di Guardo et al., 2013
D. delphis	1990	Italy	Unknown	S	CeMV	Van Bressem et al., 1993
G. melas	2006-2007	France, Spain	Epidemic	IHC, RT-PCR	DMV	Fernandez et al., 2008
G. griseus	1997, 1999	Spain	Unknown	S	CeMV	Van Bressem et al., 2001
B. acutorostrata	1993	Italy	Unknown	S	Unknown	Di Guardo et al., 1995
B. physalus	2011	Italy	Periodic mortalities	RT-PCR	DMV	Mazzariol et al., 2012

*: Abbreviations are: VI = virus isolation, IHC = immunohistochemistry, S = serology, RT-PCR = reverse-transcriptase polymerase chain reaction, PMV = porpoise morbillivirus, CeMV = cetacean morbillivirus, DMV = dolphin morbillivirus, PWMV = pilot whale morbillivirus and CeMV.

2. AIM

In order to response to main answers on the difficult relationship between the immune system of cetaceans and one of the main threats for their conservation, dolphin morbillivirus (DMV), the present study is divided in two different topics, the marine mammals immune system and the dolphin morbillivirus, in order to better investigate the two different aspects and then compared results and observation obtained.

In order to increase the knowledge on the marine mammals immune system, its morphology and the different cellular response, the fist topic was aimed to:

- Analyze the immune system of striped dolphin (*Stenella coeruleoalba*) and bottlenose dolphin (*Tursiops truncatus*), the two species more often stranded along the Italian coastline.
- Characterize the lymphocyte sub-populations using monoclonal anti-human antibodies (CD3, CD20 and HLA-DR) in paraffin embedded lymphatic tissues.
- Validate the use of these commercial monoclonal anti-human antibodies in the species under study by Western Blotting technique.
- Perform a semi-quantitative analysis on positive immunolabelling cells in the dolphins tissue samples.
- Look for a possible statistically significant correlation between the different lymphocyte sub-populations and independent variables such as species, gender, age class, regional areas and the presence of ongoing infections.

Since lymphoid organs of dolphins are one of the main targets of DMV, one of the emerging pathogens for cetaceans worldwide, and DMV is gradually increasing its host range, the other research topic was to increase the knowledge on this virus by:

- Developing a very sensitive molecular technique usable in stranded cetaceans tissues to detect DMV, necessary when RNA degradation occur rapidly after animals death.
- Investigating the impact of DMV infection in new host species;
- Analyzing the complete DMV genome sequence to underlying any change in the viral structure and its influence on the viral virulence and pathogenicity.

3. MATERIALS AND METHODS

Since the present study has two different targets, namely the characterization of immune system of marine mammals and the investigations on dolphin morbillivirus, this chapter has been divided in two different sections to give better details on the methodology used.

3.1 IMMUNE SYSTEM

3.1.1 Animals and sampling

To investigate on the marine mammals immune system study, samples of lymphatic tissue (spleen, lymph node and/or thymus) taken from 16 animals, 12 striped dolphins (*Stenella coeruleoalba*) and 4 bottlenose dolphins (*Tursiops truncatus*), were selected among the more than 300 cetaceans present in the Mediterranean Marine Mammals Tissue Bank (Table 3.1), based on the carcass conservation status and anamnestic data. Since in most of the cases, data on life history of these animals were available, age was estimated on total body length and/or on teeth microscopic examination. Furthermore, only dolphins selected stranded along the Italian coastline or died in Italian aquaria and fully necropsied were selected. In order to have a comparison with another basin with different geographical features, 11 cetaceans stranded along the Canary Island and died for ship strikes with no other pathological findings and/or molecular evidences of infection have been included in the control group (Table 3.1).

3.1.2 Microscopic and immunohistochemical (IHC) analyses

The immune system of marine mammals were examined by routine microscopic examination and immunohistochemical (IHC) analysis: lymph node and/or spleen and/or thymus were examined in the present study were fixed in 4% buffered formalin, paraffin embedded and stained for routine microscopic examination using hematoxylin and eosin.

For IHC analysis, staining was performed using an automatic immunostainer (Ventana Benchmark XT, Roche-Diagnostic), which uses a kit with secondary antibody with a horseradish peroxidase (HRP)-conjugated polymer that binds mouse and rabbit primary antibodies (ultraViews Universal DAB, Ventana Medical System). All reagents were dispensed automatically except for the primary antibody, which was dispensed by hand. They were used a monoclonal mouse anti-human CD3 (clone F7.2.38; Dako, Agilent pathology Solutions) at a dilution of 1:50, a monoclonal mouse anti-

human CD20 (Thermo Scientific) at a dilution of 1:800 both incubated for 13 minutes at room temperature and a monoclonal mouse anti-human HLA-DR, alfa-chain (clone TAL.1B5; Dako, Agilent pathology Solutions), at a dilution of 1:50 incubated for 32 minutes at room temperature (Summarized in Table 3.2) .

3.1.3 Western Blotting analysis

Total membrane protein from cells of bottlenose dolphin, striped dolphin and human lymph node samples was homogenized using Potter glass (Vetrotecnica, Italia) in 5 ml of buffer A (10 mM Tris, 150 mM NaCl, 5 mM pH 7.2 EDTA and cocktail inhibitor) and centrifuged at 10000 g for 30 minutes. The supernatant was then centrifuged at 125000 g for 1 hours and the membrane proteins were dissolved in 0.2 ml of buffer B (10 mM Tris, 150 mM pH 7.2 NaCl) and diluted 1:1 in Sample Buffer (Sigma-Aldrich, Co., St. Louis, MO, USA) and stored at −20 °C until use. Total protein concentration was determined using BCA Protein Assay Kit (Pierce Biotechnology, USA).

Protein samples separation were performed using 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli protocol (1970).

Protein (1.5µg/10µl for striped dolphin and bottlenose dolphin tissue, 0.3 µg/10µl for human tissue) was loaded in a trans-blot (Elettrofor, Rovigo, Italia) and run in running buffer (TRIS-base 25mM, Glicina 192m, Metanolo 20%; pH 8.3) at 350 V for 1 hours at 4°C.

After molecular weight separation, protein were then transferred to nitrocellulose membranes in Ponceau staining and membranes were washed in deionized water and blocked overnight at room temperature with 5% skin milk.

Dilution of CD3, CD20 and HLA-DR antibody was defined after appropriate dilution tests and cross-reaction with the secondary anti-rabbit or anti-mouse antibody horseradish peroxidase-conjugated (GAR-HRP and Gam-HRP respectively) (Table 3.3).

The primary and secondary antibody incubations were performed at room temperature in nitrocellulose membranes with 5% skin milk, for 40 minutes and 1 hours respectively. Wash steps before and after addition of secondary antibody consisted of three 10 minutes incubations in PBS buffer 0.1% Tween-20 (Sigma-Aldrich, Co., St. Louis, MO, USA).

Finally, the blots were exposed to film (GE Healthcare, UK), digitally captured using ImageScanner (Amerscham Biosciences, NJ, USA) and analyzed by ImageMaster TotalLab program (Amerscham Biosciences, NJ, USA).

3.1.4 Semi-quantitative analysis and statistical analysis

A semi-quantitative analysis was performed using a slide scanner for digital pathology (D-sight, A. Menarini diagnostic). Each IHC staining slide was scan and immunolabelled cells were counted by two operators in 10 fields at original magnification 40x, considered to be representative of the entire lymphatic tissue. The count was performed manually using an open source image processing program designed for scientific multidimensional images (ImageJ, LOCI, University of Wisconsin-Madison).

Statistical analyses were performed to find possible correlation between the different lymphocytic sub-populations expression and independent variables such as species, gender, age class, regional areas and the presence of ongoing infections.

For the statistical analysis the T-test was chosen because of the heterogeneity of the samples and the amount of data. A statistically significant threshold was set at a p-value of 0.05; a p-value less than 0.05 was considered indicative of a strong association.

Table 3.1. Data concerning the 27 cetaceans under study*.

ID	Species	Sex	Age class	Stranding place	Conserv. code	Ongoing infections
145	*Tursiops truncatus*	M	Pup	UUC	2	None
167	*Stenella coeruleoalba*	M	Adult	Collesalvetti (LI)	2	None
170	*Stenella coeruleoalba*	F	Adult	Capalbio (GR)	2	None
196	*Tursiops truncatus*	M	Adult	Cervia (RA)	2	Toxoplasma spp.
212	*Stenella coeruleoalba*	F	Adult	Livorno (LI)	2	None
214	*Stenella coeruleoalba*	F	Adult	Porto Garibaldi (FE)	3	None
218	*Stenella coeruleoalba*	M	Adult	Lido di Classe (RA)	1/2	None
221	*Stenella coeruleoalba*	M	Adult	Lido di Volano (FE)	2	None
229	*Tursiops truncatus*	M	Pup	UUC	1	None
251	*Stenella coeruleoalba*	M		Giugliano (NA)	2	Morbillivirus
255	*Stenella coeruleoalba*	F		Civitavecchia (RO)	1	None

262	*Stenella coeruleoalba*	M		Napoli (NA)	2	Morbillivirus
267	*Stenella coeruleoalba*	F	Adult	Ortoliuzzo (ME)	2	None
273	*Stenella coeruleoalba*			Salerno (SA)	2	None
327	*Stenella coeruleoalba*	M	Adult	Brancaleone Marina (RC)	1	None
343	*Tursiops truncatus*	M	Pup	UUC	1	None
CET 131	*Delphinus delphis*	M	Pup	Guía de Isora (Tenerife)	2	None
CET 151	*Stenella coeruleoalba*	M	Juvenile	La Graciosa (La Graciosa)	1	None
CET 281	*Stenella coeruleoalba*	F	Adult	Puerto del Carmen (Lanzarote)	2	None
CET 293	*Stenella coeruleoalba*	M	Adult	Arico (Tenerife)	2	None
CET 371	*Stenella coeruleoalba*	F	Adult	Arona (Tenerife)	2	None
CET 374	*Stenella coeruleoalba*	M	Adult	Playa Tebeto (Fuerteventura)	2	None
CET 406	*Delphinus delphis*	M	Pup	Santiago (Tenerife)	2	None
CET 483	*Grampus griseus*	M	Adult	Puerto del Rosario (Fuerteventura)	2	None
CET 606	*Stenella coeruleoalba*	F	Adult	Teguise (Lanzarote)	2	None
CET 616	*Stenella coeruleoalba*	F	Adult	Mogan (Gran Canaria)	2	None
CET 698	*Stenella coeruleoalba*	F	Adult	Los Giunchos (La Palma)	2	None

*: M = male; F = female; UUC = under human care

Table 3.2. Antibody used for IHC analysis

Mono/Polyclonal	Antibody name	Clone	Target cells	Antigen localization
Monoclonal Mouse	Anti-human CD3	F7.2.38	T lymphocytes	Cell membrane
Monoclonal Rabbit	Anti-human CD20		B lymphocytes	Cell membrane and cytoplasm
Monoclonal Mouse	Anti-human HLA-DR Alpha-chain	TAL.1B5	Antigen presenting cells	Cell membrane

Table 3.3. Antibody dilutions for Western Blotting protocol*.

		Anti-CD3	**Anti-CD20**	**Anti-HLA-DR**
	mw	61	33	33
Primary Ab		1:500	1:2000	1:2000
Secondary Ab GAR-HRP		1:50000	1:50000	
Secondary Ab GAM-HRP				1:8000

*: Ab = antibody; GAR-HRP = anti-rabbit antibody horseradish peroxidase-conjugated; GAM-HRP = anti-mouse antibody horseradish peroxidase-conjugated

3.2 DOLPHIN MORBILLIVIRUS

3.2.1 Immunohistochemical (IHC) analysis

IHC analysis for dolphin morbillivirus (DMV) was performed on paraffin wax embedded sections using two different primary antibodies: a murine monoclonal antibody against canine distemper virus nucleoprotein (VMRD Inc., Pullman, WA, USA) and a rabbit hyperimmune anti–rinderpest virus serum (provided by Pirbright Institute, Pirbright, UK). The slides were dewaxed for 15 minutes in xylene at room temperature, dehydrated for 5 minutes in absolute ethanol, in 90% ethanol and in 70% ethanol respectively, then washed for 10 minute in distilled water. The sections were then transferred to a 3% H_20_2 solution for 20 minutes at room temperature, washed and incubated in pH6 citrate buffer 50 ml at 95℃ for 15 minutes. The slides were washed in PBS buffer and incubated in 5% bovine albumin for 30 minutes at room temperature, then with the primary antibody for 1 hour at room temperature. The sections were at last incubated with the secondary antibody (EnVision FLEX/HRP ready to use) for 1 hour at room temperature and for 2 minutes in the diaminobenzidine (DAB) chromogen, then after washing in distilled water, specimens were briefly counterstained in Mayer hematoxylin. Slides were then washed in tap water for 3 minutes before being dehydrated through an ethanol series: 70%, 95%, and 100% for 2 minutes each.
IHC analysis were performed on available tissues (lung, brain, spleen and/or lymphoid tissues) of the 5 large whales (*BP#1* and *BP#2*, *PM#1*, *PM#2* and *PM#3*) reported in Table 3.4.

3.2.2 Tissue sampling for nested RT-PCR protocol

In order to develop a nested RT-PCR protocol for DMV detection in badly preserved cetacean carcasses and in stranded whales, tissues from 8 marine mammals showing microscopic and/or biomolecular evidence of morbilliviral infection were selected, with 4 of them having already been included in previous studies, as reported in Table 3.4 (one adult male bottlenose dolphin, *Tursiops truncatus*, one under human care adult male harbor seal, *Phoca vitulina*, two young female fin whales, *Balaenoptera physalus*) (Di Guardo et al., 2013; Mazzariol et al., 2013; Mazzariol et al., 2016). In addition, one adult female striped dolphin (*Stenella coeruleoalba*) stranded on the Tuscany coast of Italy in 2010, along with one *foetus* and two adult sperm whales (*Physeter macrocephalus*) stranded in 2014 along the Italian coastline, were investigated by means of the nested RT-PCR technique (Table 3.4). The preservation conditions of the examined animals were assessed by a veterinary pathologist on microscopic observation, thereby evaluating the magnitude

of autolytic changes occurring in lung, brain and lymphoid tissues, which are common DMV (and morbilliviral) infection's targets (Van Bressem et al., 2014).

According to standard protocols, tissues routinely collected from the aforementioned animals were represented by lung, brain, spleen and other lymphoid tissues (lymph nodes and/or tonsils). Due to field conditions, which did not always allow an immediate samples' collection and freezing, each tissue was collected separately at room temperature during the necropsy, to be subsequently transported and frozen at the end of all the procedures, approximately within 72 hours. The conservation codes of the 8 aquatic mammal carcasses investigated herein are reported in Table 1: these codes reflect the gross *post mortem* autolysis-related changes according to the time elapsed between death and necropsy (Geraci and Lounsbury, 2005); they do not reflect the microscopic autolytic changes observed in the different tissues from the animals under study.

3.2.3 Viral RNA extraction and retrotranscription for nested RT-PCR protocol

Total RNA extraction was performed with two different methodologies: (i) by pressure filtration method, using PureLink RNA Mini Kit (Ambion, Thermo Scientific) following manufacturer's instructions and (ii) using TRIzol Reagent (Ambion, Thermo Scientific) following the supplier's protocol. Briefly, tissues were placed and homogenized in 1 ml TRIzol Reagent and incubated for 5 minutes at room temperature. Then 0.2 ml of chloroform per 1 ml TRIzol Reagent was added and the mixture was vigorously shaken by hand for 15 sec and incubated at room temperature for 2-3 min. The RNA was then precipitated by mixing with isopropyl alcohol. The supernatant was removed and pellets were washed twice with 75% ethanol, to be subsequently centrifuged at < 7500 rpm for 5 min at 4ºC. The RNA pellets were air-dried and then dissolved in 40-80µL RNAase-free water. The obtained RNAs were quantified using NanoDrop 1000 (Thermo Scientific) and the corresponding cDNAs were obtained employing a previously published primer named DMV2 (Bellière et al., 2011; Table 3.5). Six micrograms of total RNA were used for the retrotranscriptase reaction, which was carried out according to manufacturer's instructions (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific).

3.2.4 Viral RNA extraction and retrotranscription for complete DMV genome study

In order to study the DMV complete viral genome previously described in a fin whale (Mazzariol et al., 2016; Table 3.5), total RNA extraction from lung, brain and spleen tissue was performed using PureLink RNA Mini Kit (Ambion, Thermo Scientific) following manufacturer's instructions. The obtained RNAs were quantified using NanoDrop 1000 (Thermo Scientific) and the corresponding

cDNAs were obtained employing a previous published primer named DMV2 (Bellière et al., 2011; Table 3.5). From six to eight micrograms of total RNA were used for the retrotranscriptase reaction carried out according to manufacturer's indications (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific).

3.2.5 Primer design and nested PCR steps

Three pairs of primers (DMV-10, DMV-11 and DMV-12; Table 3.5) tested for the first PCR step, were previously used to detect three different DMV fragments (Mazzariol et al., 2016). The primers chosen for first step of PCR (DMV-11; Table 3.5) are able to detect a 612 base pair (bp) sequence of the most conserved region of DMV haemagglutinin (H) gene (Mazzariol et al., 2016). The set of primers for the second round (DMV-13; Table 3.5) was designed based on the available DMV H gene sequence (GenBank accession number AJ608288), in order to detect a 200 bp sequence within the first 612 bp product. For the first PCR step, amplification was performed by means of a high-fidelity polymerase (Phusion Hot Start II DNA Polymerase, Thermo Scientific), with the following PCR conditions: 30 sec at 98°C; 35 cycles of 10 sec at 98°C, 30 sec at 56°C, 1 min at 72°C; 10 min at 72°C. The second PCR step was carried out using the same high-fidelity polymerase and PCR conditions, with the only change of the annealing temperature at 62°C. The nested PCR products obtained were size-separated by agarose gel electrophoresis, to be subsequently displayed in agarose gel and directly sequenced (3730xl DNA Analyzer, Thermo Scientific).

3.2.6 Primer design, PCR protocol and cloning procedures for complete DMV genome study

Fifteen pairs of primers was used to performed conventional PCR; four of these pairs and two single primers (DMV-N1 and DMV-P2, DMV-10 pair, DMV-11 pair and DMV-12pair; DMV-C and DMV-F6; Table 3.5) were previously used to detect different DMV fragments (Belliere et al., 2011; Mazzariol et al., 2016). The other set of primers (Table 3.5) were designed using Prime3 based on the available DMV gene sequence Genbank accession number AJ608288, in order to detect all the six transcription units coding the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the fusion glycoprotein (F), haemagglutinin (H) as well as two virulence factor proteins (C and V). Amplification was performed using a high-fidelity polymerase (Phusion Hot Start II DNA Polymerase, Thermo Scientific), with the following PCR conditions: 30 sec at 98°C; 35 cycles of 10 sec at 98°C, 30 sec at 58°C, 1 min at 72°C; 10 min at 72°C. The PCR products obtained were size-separated by agarose gel electrophoresis, to be then displayed in agarose gel (3730xl DNA Analyzer, Thermo Scientific).

The PCR product obtained from lung, cerebral and spleen cDNA were purified, cloned into plasmid

vector PCR-Blunt II TOPO (Thermo Scientific) according to the manufacturer's instructions, and then sequenced. Three sequences from lung, cerebral and spleen plasmidic colonies were analyzed. Programs in the DNASTAR Lasergene software package (http://www.dnastar.com/t-dnastar-lasergene.aspx) were used to edit, assemble, and translate sequences.

3.2.7 Secondary structure prediction for complete DMV genome study

In our research, we analyzed primary structures of gene N, gene M, gene F, gene H and genes P/V/C of DMV protein sequence, in order to identify differences in secondary structures from DMV reported sequence (Genbank Acc. No. AJ608288) and our sequences. Each protein sequence was subjected to consensus secondary structure prediction using PSIPRED (Jones, 1999) .

3.2.8 Nucleotide and amino-acid sequence comparison for complete DMV genome study

Accounting for their coding nature, sequences of DMV genes (i.e. H, F, M and N genes) collected from fin whales (present study) and from a striped dolphin (Genbank Acc. No. AJ608288) were aligned at amino acid level using the MAFFT algorithm implemented in TransaltorX and then back-translated to nucleotide. Differences between the two strains as well as their effect (I.e. synonymous or non-synonymous mutations) were recorded.

3.2.9 Homology modelling for complete DMV genome study

To evaluate the morphological changes caused by non-synonymous mutations the tertiary and/or quaternary structure of relevant proteins were evaluated and compared between strains collected from different host.

*S*equences of DMV genes (i.e. H, F, M and N genes) collected from fin whales (present study) and from striped dolphin (Genbank Acc. No. AJ608288) were translated at amino acid level and the best protein template for which tertiary or quaternary structure has been experimentally determined was searched using the SWISS-MODEL web server (Arnold et al., 2006).

The same program was used for modeling the protein structure through an homology modeling approach (Biasini et al., 2014). For each protein the structural alignment was performed using the MatchMaker method implemented in Chimera. This approach performs a fit after automatically identifying which residues should be paired using both sequence and secondary structure, allowing similar structures to be superimposed even when their sequence similarity is low. Similarly, animation were created using the morph conformation tool implemented in the same software (Petterson et al., 2004).

Table 3.4. Data concerning the eight aquatic mammals under study.*

Animal number	Species	Sex	Age class	Conservation code§	Tissue
TT#1	*Tursiops truncatus*	M	Adult	1	Lung
					Brain
					Spleen
					Lymph node
PV#1	*Phoca vitulina*	M	Adult	1	Lung
					Brain
					Lymph node
BP#1	*Balaenoptera physalus*	F	Juvenile	2	Lung
					Spleen
					Lymph node
BP#2	*Balaenoptera physalus*	F	New born	2	Lung
					Brain
					Spleen
SC#1	*Stenella coeruleoalba*	F	Adult	2	Lung
					Lymph node
PM#1	*Physeter macrocephalus*	F	Adult	2	Lung
					Brain
					Spleen

PM#2	*Physeter macrocephalus*	F	Adult	2	Lung
PM#3	*Physeter macrocephalus*	F	Fetus	2	Lung
					Kidney

*: RT-PCR = reverse transcription polymerase chain reaction; Y = yes; N = no; ND = not done
§: Carcass conservation *status* reflects informations according to Geraci and Lounsbury (2005); it does not correspond to the autolysis degree observed during microscopical investigations.

Table 3.5. RT-PCR (conventional RT-PCR and nested RT-PCR) primer sets.*

Primer name	***nt* position (referred to AJ608288)**	**5′→3′ sequence (sense)**	**Fragment length, bp**	**Annealing temperature**
DMV-2	15702–15684	ATHCCCAGCTTTGTCTGGT	cDNA production	
DMV-1F	72-92	TCAATTGGCACAGGATTTGG	474	56°C
DMV-1R	545-525	CCAATGGGTTCCTCTGGTGT		
DMV-2F	501-521	TCTATTCAAGCAGGGGAGGA	622	56°C
DMV-2R	1122-1102	TCGGCTGTGATCCCTAGTTC		
DMV-N1	1203-1222	CAAGAGATGGTCAGGAGATC	1358	56°C
DMV-P2	2541-2521	GACAGGTGGTGCAACCCGAC		
DMV-C	2132-2152	ATGTTTATGATCACGGCGGT	769	56°C
DMV-4R	2900-2880	AGGTGGCCTTCGATAGTTGA		
DMV-5F	2439-2459	ACCAATTCCAACCTCAGTGC	716	56°C
DMV-5R	3154-3134	ATCCCACAGCAGAGCTCATT		
DMV-6F	5178-5198	TGGTCGTCAACATTGAGTCAC	690	56°C
DMV-F6	5852-5835	CGCAAGACAGCTGGTGC		
DMV-7F	5684-5704	GCCCTTCATCAGTCCATCAT	667	56°C
DMV-7R	6334-6314	ATTGTTGGAGCAACGGACTC		
DMV-8F	6147-6167	CAGAGGTCAAGGGGGTGATA	700	56°C
DMV-8R	6827-6807	CGACAGTGCCTCCTACAACA		

DMV-9F	6482-6502	GGCACCATAATTAGCCAGGA	778	56°C
DMV-9R	7240-7220	CCTGCAATGGCAAGTAGTCC		
DMV-10F	7206-7226	GGGTGTGCTAGCCGTTATGT	718	56°C
DMV-10R	7904-7884	TTCGTCCTCATCAATCACCA		
DMV-11F	7799-7819	CCGAACCTGATGATCCATTT	612	56°C
DMV-11R	8411-8391	CGTAAATGTCCATCCCTGCT		
DMV-12F	8290-8310	AACCGGATCCCAGCTTATG	800	56°C
DVM-12R	9070-9050	CCAGGTGCACTTCAGGGTAT		
DMV-13F	8052-8072	CATCATAGGGGGTGGTTTGA	200	62°C
DMV-13R	8232-8212	GGGGTGGTCTACTCTTGCAC		
DMV-14F	3037-3057	CCAGCAGTCGAGAGAAATCC	723	56°C
DMV-14R	3759-3739	TCTCATTTAACCCCGCTGTC		
DMV-15R	3464-3484	CTGGGATGTCAAGGGGTCTA	612	56°C
DMV-15F	4075-4055	GCCTGTGGGTCTCTCATCAT		
DMV-16R	3920-3942	CAGACTCTCAGACAATGGATGC	586	56°C
DMV16F	4505-4485	GCTCTGTTGATTCTGCTGGA		

*: nt = nucleotide; bp = base pair

4. RESULTS

As already done for the previous chapters, in order to give clear an detailed information on the results obtained from the performed investigations, studies on the immune system of marine mammals and those on dolphin morbillivirus are treated separately.

4.1 IMMUNE SYSTEM

The investigation on the marine mammals immune system includes a total of 27 dolphins: 20 striped dolphins (*Stenella coeruleoalba*), 4 bottlenose dolphins (*Tursiops truncatus*), 2 common dolphins (*Delphinus delphis*) and 1 Risso's dolphin (*Grampus griseus*); considering the gender, 10 females (37%) and 16 males (59.3%) and for one animal sex determination was not available. About the 22% of the examined animals were pup or sub-adult (6 cetaceans) while the other 63% (17 animal) were adults; for 4 animal age class determination was not given.
About half of the marine mammals under study came from the Mediterranean basin (13 animals, 48,1%), the 40% stranded along the Canarian coastline; 3 animals were selected from those died under human care.

4.1.1 Microscopic and immunohistochemical (IHC) analyses

The architecture of the lymph node varies depending on location: the major differences involve the amount of muscle in the capsule and the trabeculae. The somatic nodes have very little smooth muscle in this location, on the contrary, in the visceral nodes examined in the study (mainly the mediastinic or the pre-scapular lymph nodes) smooth muscle encapsulates the node itself and in addition to extending along trabeculae, forms an interlacing network throughout the node.
The cetacean spleen is composed of white pulp, consisting of lymphoid nodules developed at arterial terminals, evenly distributed throughout the red pulp. Lymphoid nodules are composed of small to medium-sized lymphocytes. Germinal centers may be identified as white granules on gross inspection, but as they reflect a reactive state, they are inconstant and often absent.
Observation of microscopic examination carried out on the lymphoid organs of the selected animals are summarized in Table 4.1. A total of 11 marine mammals presented normal lymphoid tissues, 11 showed hyperplastic, while the organs of 5 animals were evaluated to be hypoplastic.

In order to evaluate immunohistochemically different leuckocytes population anti-human CD20 and anti-human CD3 antibodies (to evaluate B and T lymphocytes respectively) and the major histocompatibility complex class II (MHC-II) anti-human HLA-DR antibody has been selected. Their cross-reactivity was confirmed in striped dolphin and bottlenose dolphin lymphatic tissues (lymph node, spleen and thymus). More in detail, positive immunostaining for anti-human CD20 antibody was evident within lymphocytes presented in the germinal center, in the mantle and marginal zone, forming the follicle (Figure 4.1). Positive immunostaining reaction for anti-human CD3 was detected in lymphocytes localized in the para-cortical zone (Figure 4.2). The medullary area was formed by both CD20 and CD3 positive immunolabelled cells. Positive immunostaining cells to anti-human HLA-DR antibody were detected in all the lymph node structure, where cell presenting the antigen were distributed (plasma cells, macrophages and lymphocytic cells) (Figure 4.3).

The cetacean thymus follows the typical mammalian plan with a cortex, medulla, and Hassall's corpuscles. The presence of T lymphocytes has been demonstrated using the monoclonal anti-human CD3 antibody, forming the entire organs parenchyma. Randomly distributed cell clusters lacked MHC class II expression in the thymus.

4.1.2 Western Blotting analysis

The three well-defined bands, with molecular weight of 61, 33 and 33 kDa represent the specific antibodies against CD3, CD20 and HLA-DR antigen, respectively (Fig. 4.4). CD3, CD20 and HLA-DR antigens bands were identified in lymph node tissue of men (Hu), striped dolphin (ST) and bottlenose dolphin (TT).

4.1.3 Semi-quantitative analysis and statistical analysis

Results of the semi-quantitative analysis in cetaceans lymphoid organs are summarized in Table 4.2. The average number of B lymphocytes (CD20 antibody positively staining), T lymphocytes (CD3 antibody positively staining) and cells presenting the MHC-II membrane antigen was higher in lymph nodes than in spleen. As anticipate during the IHC qualitative analysis, thymus did not presented lymphocytes positively staining at the CD20 antibody.

Statistical analysis was attempted to consider possible correlations among the different lymphocytic sub-populations expression and independent variables such as species, gender, age class, provenience and the presence of ongoing infections: no strong correlation (p-value less than 0.05) were obtained; the only value that approaches a strong association was that obtained comparing the

different expression of anti-CD20 positive staining cells (B lymphocytes) in normal and hypoplastic lymph nodes (0.09) .

No other factors were influenced the different lymphocytic expression in the cetaceans under study.

Table 4.1. Details of lymphoid organs histological analysis.

	Total number of animal	Animal with ongoing diseases	*Stenella coeruleoalba (20 animal)*	*Tursiops truncatus (4 animals)*	Other species *(3 animals)*	Mediterranean basin	Atlantic basin
Normal histology	11	0 (11)	9 (11)	1 (11)	1 (11)	6 (11)	4 (11)
Hyperplastic condition	11	1 (11)	6 (11)	3 (11)	2 (11)	3 (11)	6 (11)
Hypolastic condition	5	2 (5)	4 (5)	1 (5)	0(5)	4 (5)	1 (5)

Table 4.2. Number of immunoreactive cells in cetaceans lymphoid organs.

Tissue	CD3*	CD20*	HLA-DR*
Lymp node	2391.72 ± 1053,96	2398.64 ± 965,97	2158.64 ± 808.91
Spleen	1293.67 ± 877.45	1524.17 ± 336.99	1158.17 ± 438.77
Thymus	2570.67 ± 1694.94	0	3181 ± 1587.98

*: Results expressed as mean ± standard deviation of immunoreactive cells per organ.

Figure 4.1. Striped dolphin ID 212 (*Stenella coeruleoalba*), lymph node, positive cell membrane staining of CD20 antigen. Mayer hematoxylin counterstained; original magnification 40X.

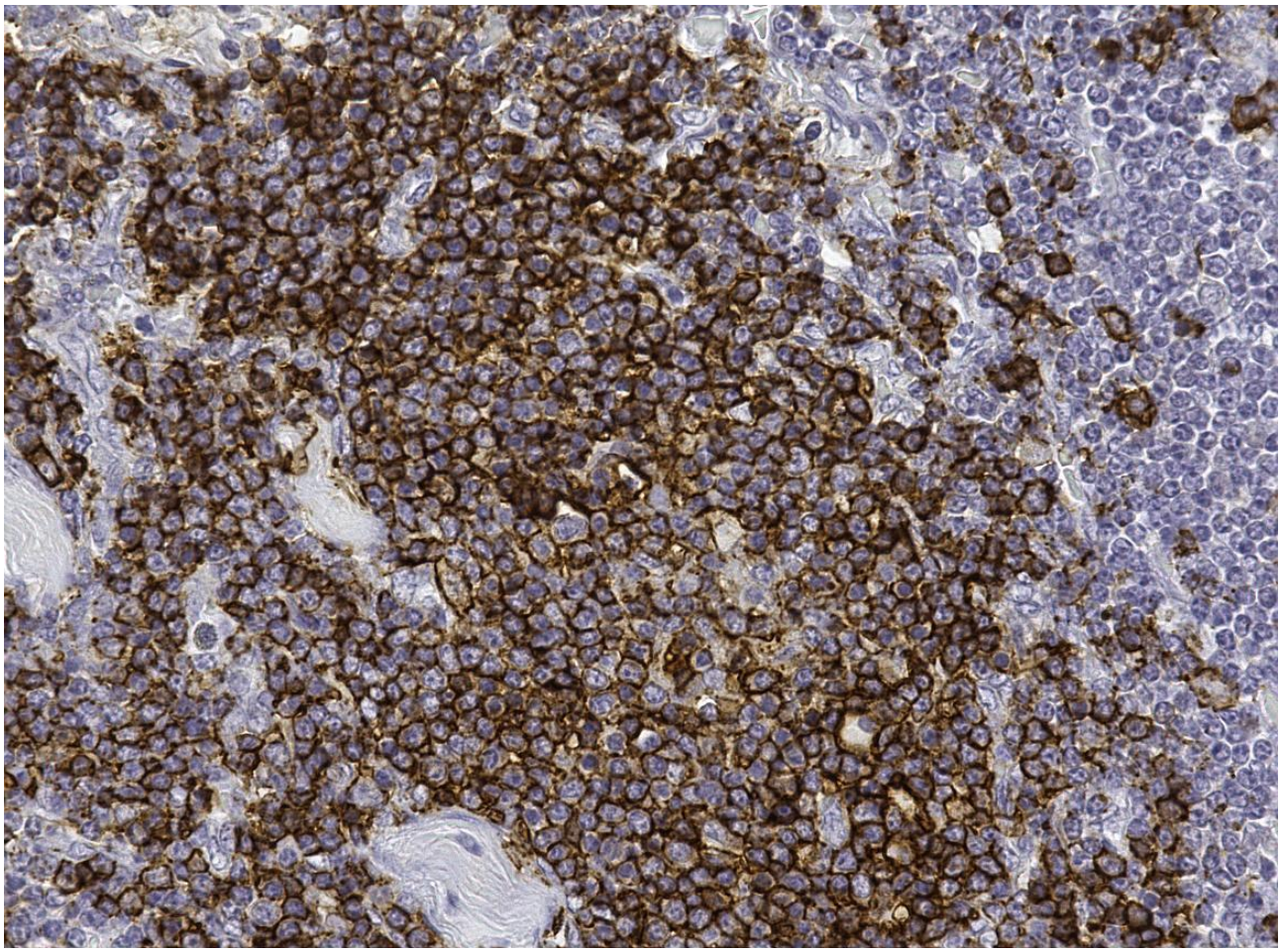


Figure 4.2. Striped dolphin ID 212 (*Stenella coeruleoalba*), lymph node, positive cell membrane staining of CD3 antigen. Mayer hematoxylin counterstained; original magnification 40X.

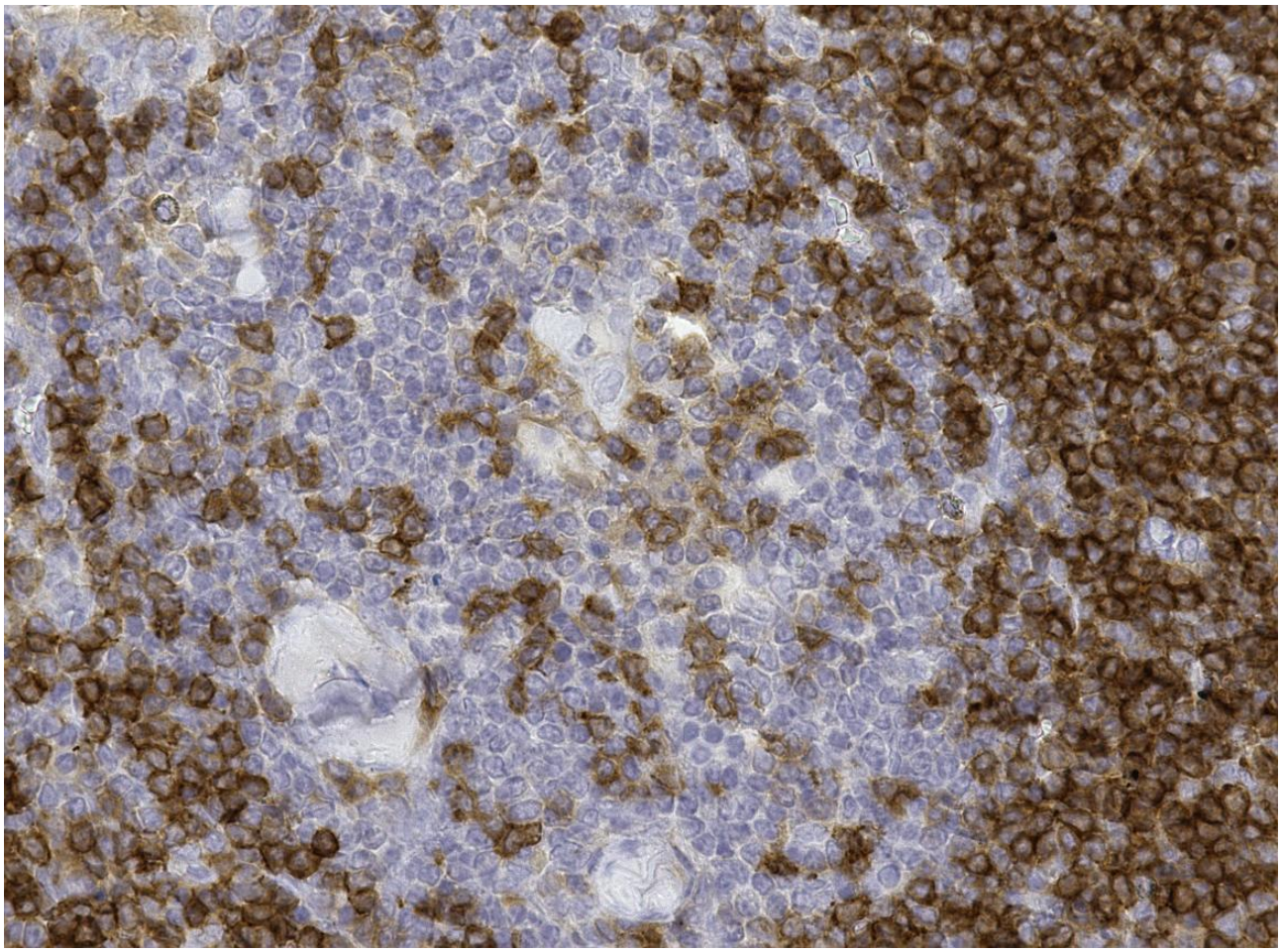


Figure 4.3. Striped dolphin ID 212 (*Stenella coeruleoalba*), lymph node, positive cell membrane staining of HLA-DR antigen. Mayer hematoxylin counterstained; original magnification 40X.

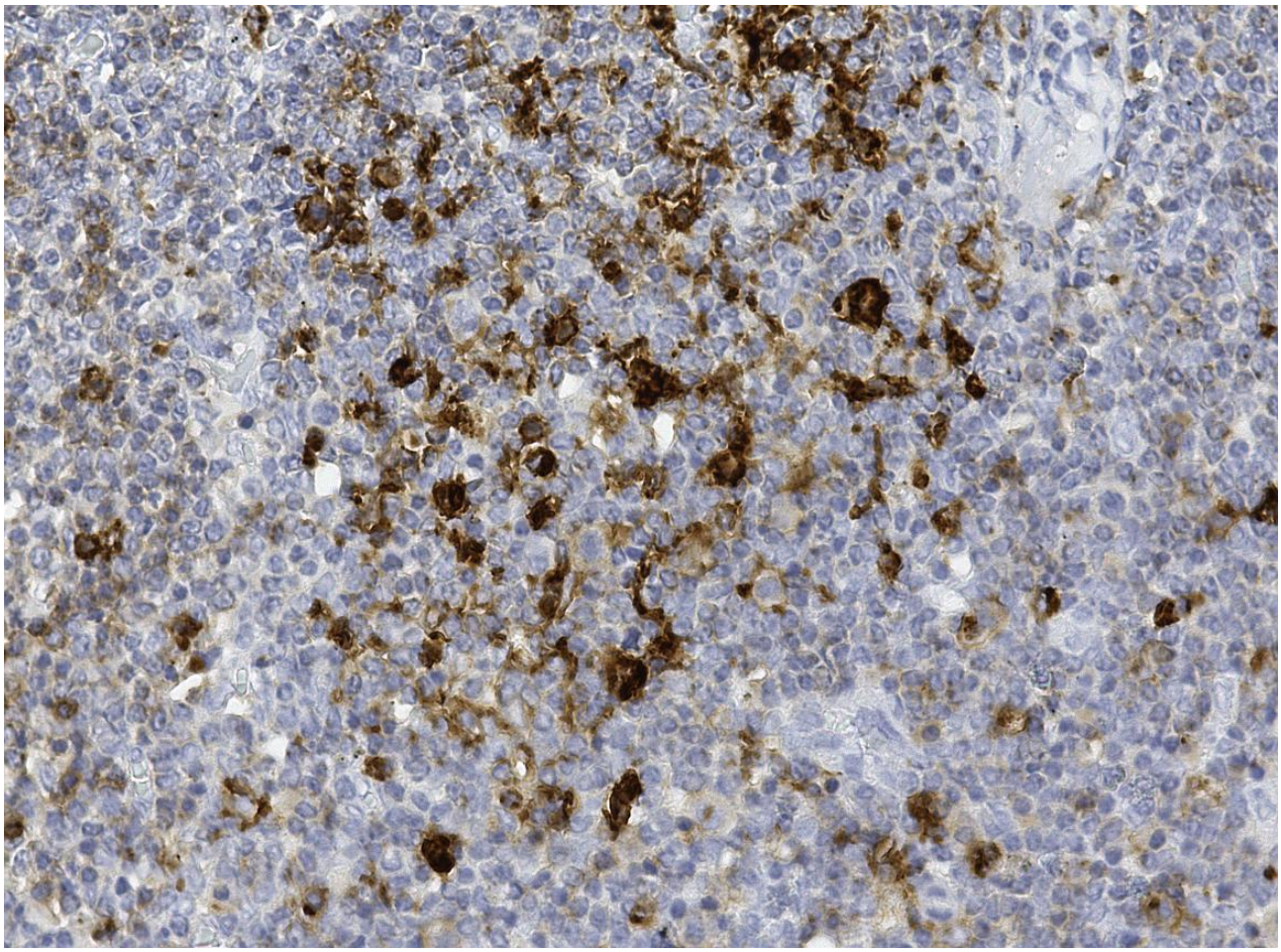
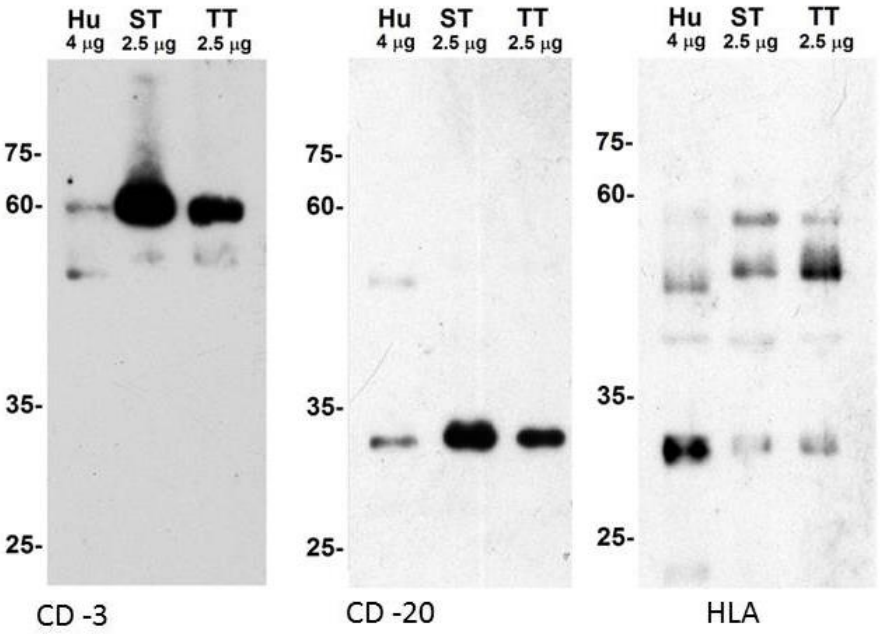


Figure 4.4. The three well-defined bands, with molecular weight of 61, 33 and 33 kDa represent the specific antibodies against CD3, CD20 and HLA-DR antigen, respectively.* CD3, CD20 and HLA-DR antigens bands were identified in lymph node tissue of men (Hu), striped dolphin (ST) and bottlenose dolphin (TT).



*: Hu = lymph node tissue of men; ST = lymph node tissue of striped dolphin (*Stenella coeruleoalba*); TT = lymph node tissue of bottlenose dolphin (*Tursiops truncatus*).

4.2 DOLPHIN MORBILLIVIRUS

4.2.1 Immunohistochemical (IHC) analyses

Circulating monocytes and tissue macrophages from the fin whale's (*BP#2*) brain and thymus were positively stained by the hyperimmune rabbit anti-Rinderpest Virus (RPV) antiserum (Institute for Animal Health, Pirbright, Surrey, England, UK) (Figure 4.5).

IHC evidence of DMV nucleoprotein (N) antigen (murine monoclonal anti CDV antibody) was observed in macrophages and follicular dendritic-like cells from the spleen's white pulp in the youngest female (*PM#1*), as well as in monocytes circulating within splenic blood vessels from the same animal (Figure 4.6).

4.2.2 Extraction methods

In this study, RNA extractions were performed by two different protocols (PureLink RNA Mini Kit and Trizol). Each of the 2 extraction methods yielded sufficient RNA amounts for obtaining positive results in a nested RT-PCR for DMV. However, the RNA extracted by the TRIzol Reagent constantly had the highest yield (400-4000 ng/microL), while the RNA extracted by the PureLink RNA Mini Kit had the lowest one (15-80 ng/microL), but the highest A260/A280 ratio for almost all samples. After the first PCR step, the PCR products from samples extracted using the PureLink RNA Mini Kit showed, with all the sets of primers and for all the samples, a more intense signal after separation by agarose gel electrophoresis.

4.2.3 Nested PCR

Nested PCR was performed on all tissue samples under investigation (Table 3.4). DMV-11 primers pair was used for the first step of PCR and DMV-13 for the second round (Table 3.5 and Figure 4.7). A sample was considered positive for DMV when it generated PCR products of the expected size of 200 bp and sequencing of the obtained fragments demonstrated that those PCR products shared 100% sequence homology with DMV (GenBank Acc. No. AJ608288). The DMV 200 bp sequence isolated from the sperm whales was registered in GenBank (Acc. No. KU886570).

As shown in Figure 4.8, positive results were obtained after application of the nested RT-PCR protocol herein reported on the 21 tissue samples under investigation (Table 4.3): *Tursiops truncatus*#1 (TT#1) brain; *Phoca vitulina*#1 (PV#1) lung, brain and lymph node; *Balaenoptera physalus*#1 (BP#1) lung and lymph node; BP#2 lung, brain and spleen; *Stenella coeruleoalba*#1 (SC#1) lung and lymph node; *Physeter microcephalus*#1 (PM#1) brain and spleen; PM#2 lung;

PM#3 lung and kidney. The same data were obtained using both RNAs extracted with PureLink RNA Mini Kit (Ambion, Thermo Scientific) and with TRIzol Reagent (Ambion, Thermo Scientific).

In order to prove the increased sensitivity of this new technique, we performed an analysis of low-titer dilution series of a positive sample (TT#1 brain tissue) using the nested RT-PCR primers herein investigated and DMV-N1 and DMV-P2 primers, as previously reported by Bellière et al. (2011) and by Mazzariol et al. (2016) (product size:1358 bp). To ensure that PCR products were authentic and not the result of cross-contamination, the number of amplification cycles was reduced to 25 for the second nested PCR step and for the conventional PCR. In conventional PCR, the cDNA lowest detection limit in agarose gel electrophoresis was defined as 1:200 dilution, while in the second step of nested PCR the lowest detection limit was 1:400000 dilution (Figure 4.9). Direct sequencing of 1:5000, 1:50000 and 1:100000 dilution of nested PCR second step products was performed to confirm their identity to DMV genome sequences.

4.2.4 Complete DMV genome study

DMV genome was detected in brain, lung and spleen of the newborn fin whale (BP#2, Table 3.4). Sequences of 5 lung, 4 cerebral and 5 spleen plasmidic colonies were analyzed. The conventional RT-PCR technique associated with viral cloning using plasmid vector allowed the identification of the entire viral genome. The sequence of each single gene was deposited in Genbank as shown below: complete N gene (1573 bp) provisional Acc. No. KU977449, complete P/V/C gene (1521 bp) provisional Acc, No. KU977450, complete M gene: (1008 bp) provisional Acc. No. KU977451, complete F gene (1659 bp) provisional Acc. No. KU977452 and complete H gene (1814 bp) provisional Acc. No. KU977453.

Afterwards each sequence was compared to the DMV complete genome isolate in the 1990's outbreak in a bottlenose dolphin (Acc. No. AJ608288): these fragments showed a sequence homology between 99.10% and 99.80%.

4.2.5 Secondary structure prediction for DMV complete genome study

PSIPRED was used to predict the secondary structure of gene N, gene M, gene H, gene F and gene P/V/C reported in literature (GenBank accession number AJ608288) and of the corresponding sequences identified in our sample.

The observed secondary structure were the same for gene M and gene H: the secondary sequence structures of the two proteins show a high degree of similarity in terms of helices and β-sheets in

our sequences as in reported ones. On the contrary the gene secondary structure gene F, gene N and gene P/V/C show difference between our and predicted sequences.

Gene F of our fin whale showed a greater amount of helices in comparison with the gene F reported in literature, in particular from residues 70 and 100 of the protein.

Gene N of our fin whale showed a high degree of similarity in terms of helices and β-sheets from the reported gene F. Moreover, fin whale gene F showed differences in particular in the disposition of boundary amino-acids.

Gene P/V/C of our fin whale showed a greater amount of helices in comparison with the gene F reported in literature, in particular from residues 185 to 275 of the protein. Moreover the sequence was different also for the amount of beta-sheets and for the disposition of boundary amino-acids.

4.2.6 Nucleotide and amino-acid sequence comparison for DMV complete genome study

Differences nucleotide and amino-acid pairwise raw distance between fin whales and bottlenose dolphin strain are reported in Table 4.4. Briefly, the genetic and amino-acidic distance were 0.01and 0.009 for F gene, 0.002 and 0.006 for M gene, 0.009 and 0.008 for N gene, 0.007 and 0.01 for the P/V/C gene and 0.006 and 0.003 for the H gene. Five out 16 substitution were non-synonymous in the F gene, 2 out 2 in the M gene, 4 out 14 in the N gene and 1 out 9 in the H gene.

Reliable (i.e. experimentally determined) structural templates were identified for the M,N,H and F proteins and consequently the tertiary/quaternary structure was predicted only for these proteins. Particularly, based on the available templates, the following regions of each protein were consistently modeled: F protein (aa 26-487), M protein (aa 20-326), N protein (aa 3-401) and H protein (157-603).

Non-synonymous changes were located in nucleus of the H (amino-acid 451) and M (amino-acids 59-190) proteins while were exposed on the protein surface of the F protein (amino-acids 52, 151,154 and 161) or were part of its signal peptide (amino-acid 3) (Figure 4.10). The only amino-acid change modeled in the N protein (amino-acid 21) was located in the inner part of the capsid (Figure 4.10). Unfortunately, amino-acidic changes located in position 436, 462 and 509 of the same protein were located in regions that could not be confidently modeled by homology modeling.

Table 4.3. Positive results obtained after application of the nested RT-PCR protocol reported on the 21 tissue samples under investigation (Table 3.4).

Animal number	Species	Tissue	Previous diagnostic tecnique	Positivity (Sequenced: Y/N)	Positivity to nested RT-PCR
TT#1	*Tursiops truncatus*	Lung	RT-PCR (Di Guardo et al., 2013)	Negative	Negative
		Brain	RT-PCR (Di Guardo et al., 2013)	Positive (N)	Positive
		Spleen	RT-PCR (Di Guardo et al., 2013)	Negative	Negative
		Lymph node	RT-PCR (Di Guardo et al., 2013)	Negative	Negative
PV#1	*Phoca vitulina*	Lung	RT-PCR (Mazzariol et al., 2013)	Positive (Y)	Positive
		Brain	RT-PCR (Mazzariol et al., 2013)	Positive (Y)	Positive
		Lymph node	RT-PCR (Mazzariol et al., 2013)	Positive (Y)	Positive
BP#1	*Balaenoptera physalus*	Lung	RT-PCR (Di Guardo et al., 2013)	Negative	Positive
		Spleen	RT-PCR (Di Guardo et al., 2013)	Positive (N)	Negative
		Lymph node	RT-PCR (Di Guardo et al., 2013)	Positive (N)	Positive

BP#2	*Balaenoptera physalus*	Lung	RT-PCR and Cloning (Mazzariol et al., 2016)	Positive (Y)	Positive
		Brain	RT-PCR and Cloning (Mazzariol et al., 2016)	Positive (Y)	Positive
		Spleen	RT-PCR and Cloning (Mazzariol et al., 2016)	Positive (N)	Positive
SC#1	*Stenella coeruleoalba*	Lung	ND	ND	Positive
		Lymph node	ND	ND	Positive
PM#1	*Physeter macrocephalus*	Lung	ND	ND	Negative
		Brain	ND	ND	Positive
		Spleen	ND	ND	Positive
PM#2	*Physeter macrocephalus*	Lung	ND	ND	Positive
PM#3	*Physeter macrocephalus*	Lung	ND	ND	Positive
		Kidney	ND	ND	Positive

Table 4.4. Differences nucleotide and amino-acid variations in each DMV single gene between fin whale (*Balaenoptera physalus*) and bottlenose dolphin (*Tursiops truncatus*) strain (GenBank Acc. No. 608288).

Nucleotide bases in AJ608288	**Nucleotide bases in fin whale**	**AA in AJ608288**	**AA in fin whale**	**AA position**
GENE N				
ATT	ACT	Ile	Thr	21
GTA	GTG	Val	Val	36
TAT	TAC	Tyr	Tyr	63
GAG	GAA	Glu	Glu	151
GAA	GAG	Glu	Glu	161
GCT	GCC	Ala	Ala	184
CTA	TTA	Leu	Leu	271

TCT	TCC	Ser	Ser	298
ATT	ATC	Ile	Ile	397
GCA	ACA	Ala	Thr	437
ACA	GCA	Thr	Ala	462
CGT	CAT	Arg	His	509
CAC	CAT	His	His	514
GAT	GAC	Asp	Asp	520
GENE P/V/C				
GAG	GAA	Glu	Glu	103
GCG	GAG	Ala	Glu	119
GGC	GGT	Gly	Gly	134
AAT	AGT	Asn	Ser	163
CTA	TTA	Leu	Leu	210
AAG	AAA	Lys	Lys	211
ATG	CTG	Met	Leu	313
CCC	CCT	Pro	Pro	396
CGA	CGG	Arg	Arg	479
GENE M				
TTA	GTA	Leu	Val	59
TTA	TCA	Leu	Ser	191
GENE F				
GCC	GCT	Ala	Ala	2
GCT	TCT	Ala	Ser	3
ACA	ACG	Thr	Thr	40
AAC	AGC	Asn	Ser	52
GTC	GTT	Val	Val	89
AAT	AGT	Asn	Ser	151
ACA	GCA	Thr	Ala	154
CAA	CGA	Gln	Arg	162
CCA	CCG	Pro	Pro	190
TTG	TTA	Leu	Leu	204
ATC	ATA	Ile	Ile	328
GTG	GTA	Val	Val	342
AAA	AAG	Lys	Lys	412
CTG	CTA	Leu	Leu	512
GTA	GTG	Val	Val	514
GENE H				
GGA	GGG	Gly	Gly	57
CTT	CTC	Leu	Leu	74
AAT	AAC	Asn	Asn	77
GTT	GCT	Val	Ala	164
CGT	CGA	Arg	Arg	262
GGG	GGA	Gly	Gly	324
GCC	ACC	Ala	Thr	451

CTT	CTC	Leu	Leu	510
GTT	GTC	Val	Val	535

Figure 4.5. Mayer hematoxylin counterstained tissue samples from the newborn fin whale (*Balaenoptera physalus*, *BP#2*). A) Brain tissue showing positive immunostaining for *Morbillivirus* sp. antigen in macrophages in the meningeal space. B) Thymus showing positive immunostaining for *Morbillivirus* sp. antigen in thymocytes and macrophages. For both samples, *Morbillivirus* sp. was detected by IHC analysis, using a rabbit hyperimmune anti–rinderpest virus serum (provided by Pirbright Institute, Pirbright, UK). Original magnification ×40.

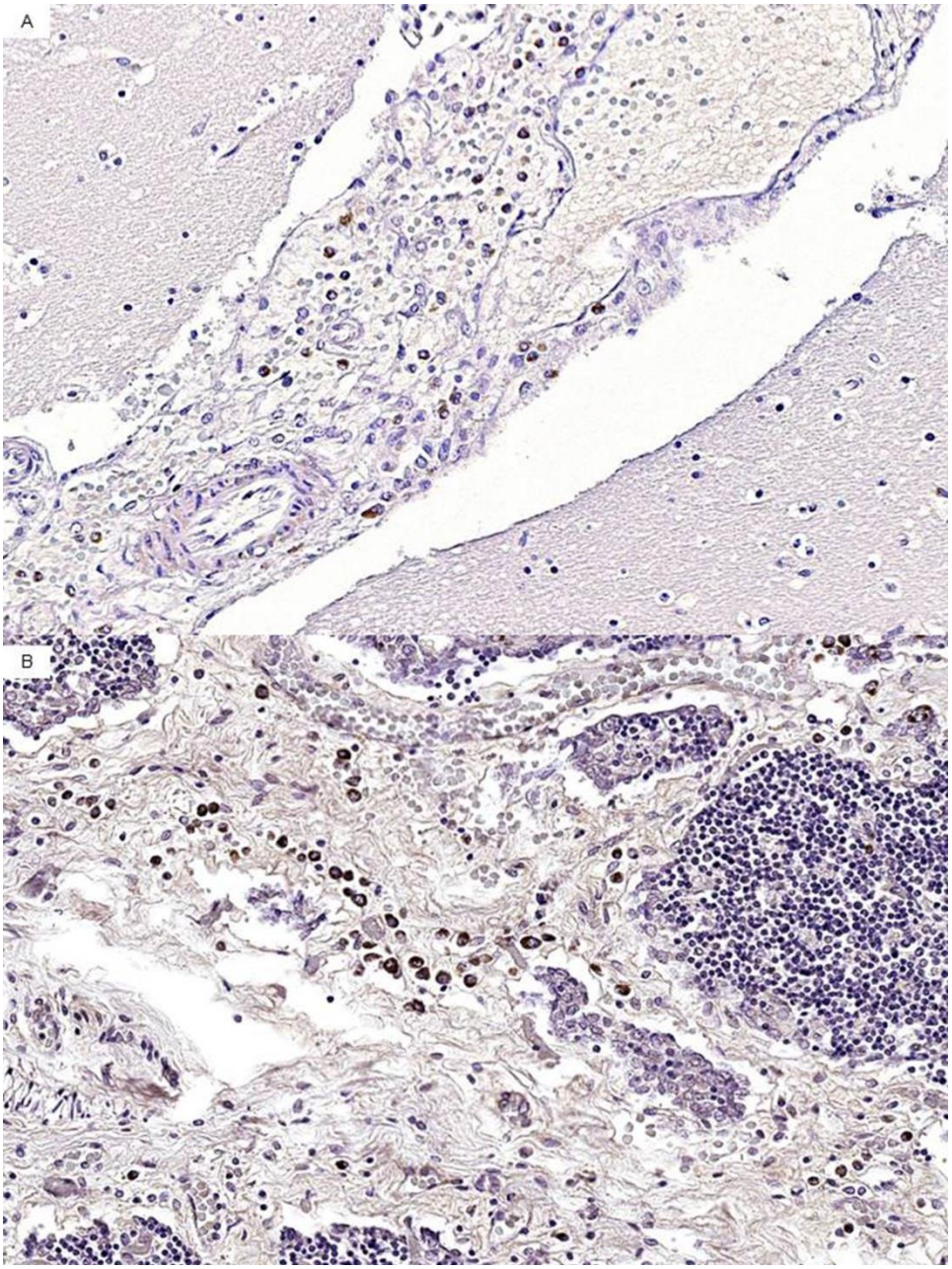


Figure 4.6. Sperm whale (*Physeter macrocephalus*; *PM#3*). Spleen. Positive immunostaining for morbilliviral antigen is shown in monocytes within vascular *lumina* and in follicular dendritic-like cells in the splenic white pulp. IHC for *Morbillivirus* with a murine monoclonal antibody against canine distemper virus (CDV) nucleoprotein (VMRD Inc., Pullman, WA, USA). Mayer's hematoxylin counterstain. Final magnification 20x.

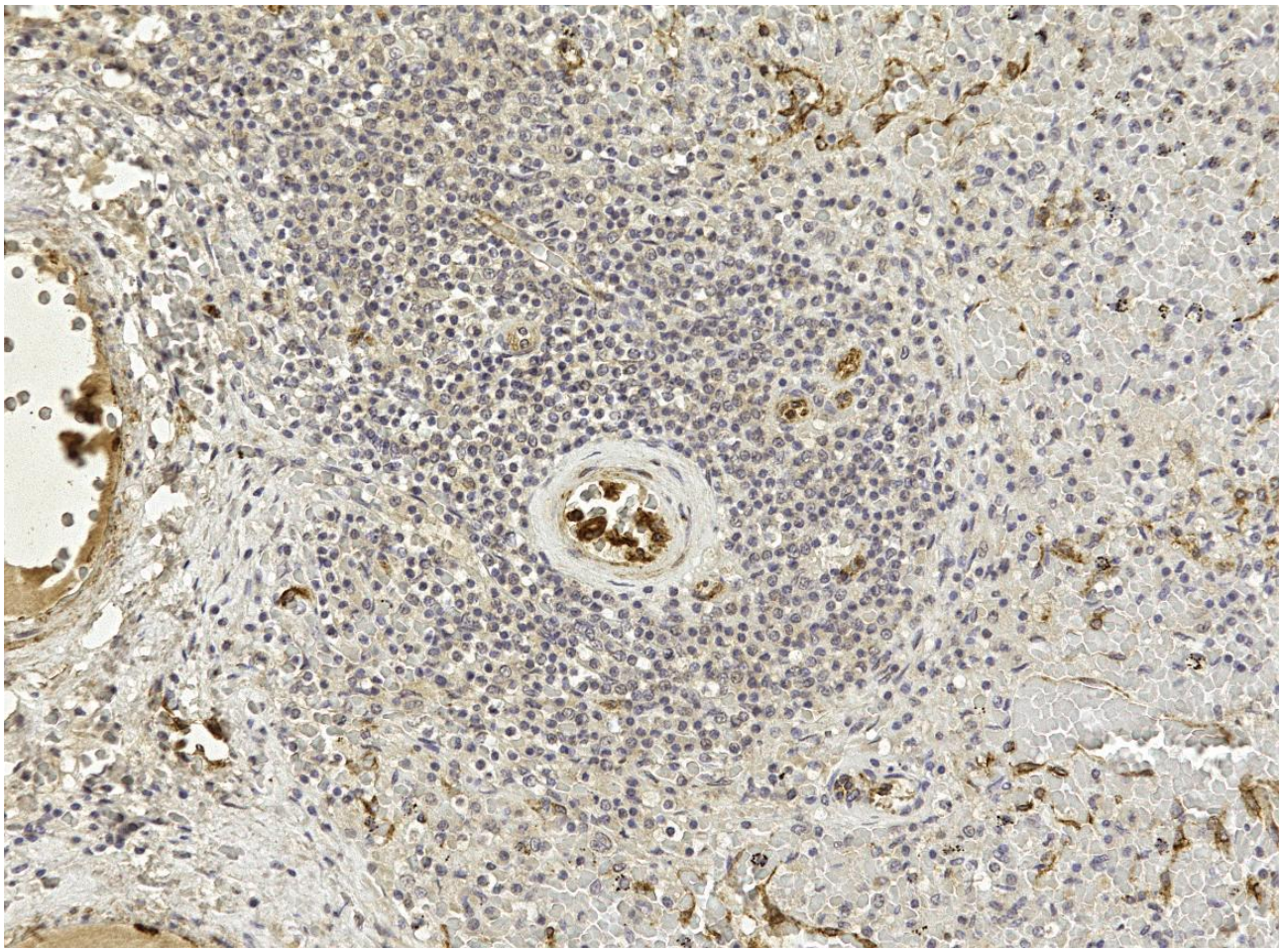


Figure 4.7. Nested RT-PCR for DMV. RNA was extracted using the PureLink RNA Mini Kit. The cDNAs were prepared using DMV2 primer pair. Agarose gel (a) and electropherogram results (b).
(a) M: DNA marker, 100 bp (DNA Ladder, Invitrogen); DMV-11F and DMV-11R primer pairs (Lanes 1 and 2) and second round of nested PCR using DMV-13F and DMV-13R primer pairs (Lanes 4 and 5). *Tursiops truncatus*#1 (*TT#1*) lung sample (Lanes 1 and 4) and *Physeter macrocephalus*#1 (*PM#1*) spleen sample (Lanes 2 and 5). Lanes 3 and 6: PCR negative controls.
(b) Electropherogram obtained from the PCR product obtained in line 5. Sequencing of the 200 bp fragment obtained from *PM#1* spleen using DMV-13F and DMV-13R primer pairs in the second step of nested PCR. Genbank Acc. No. KU886570.

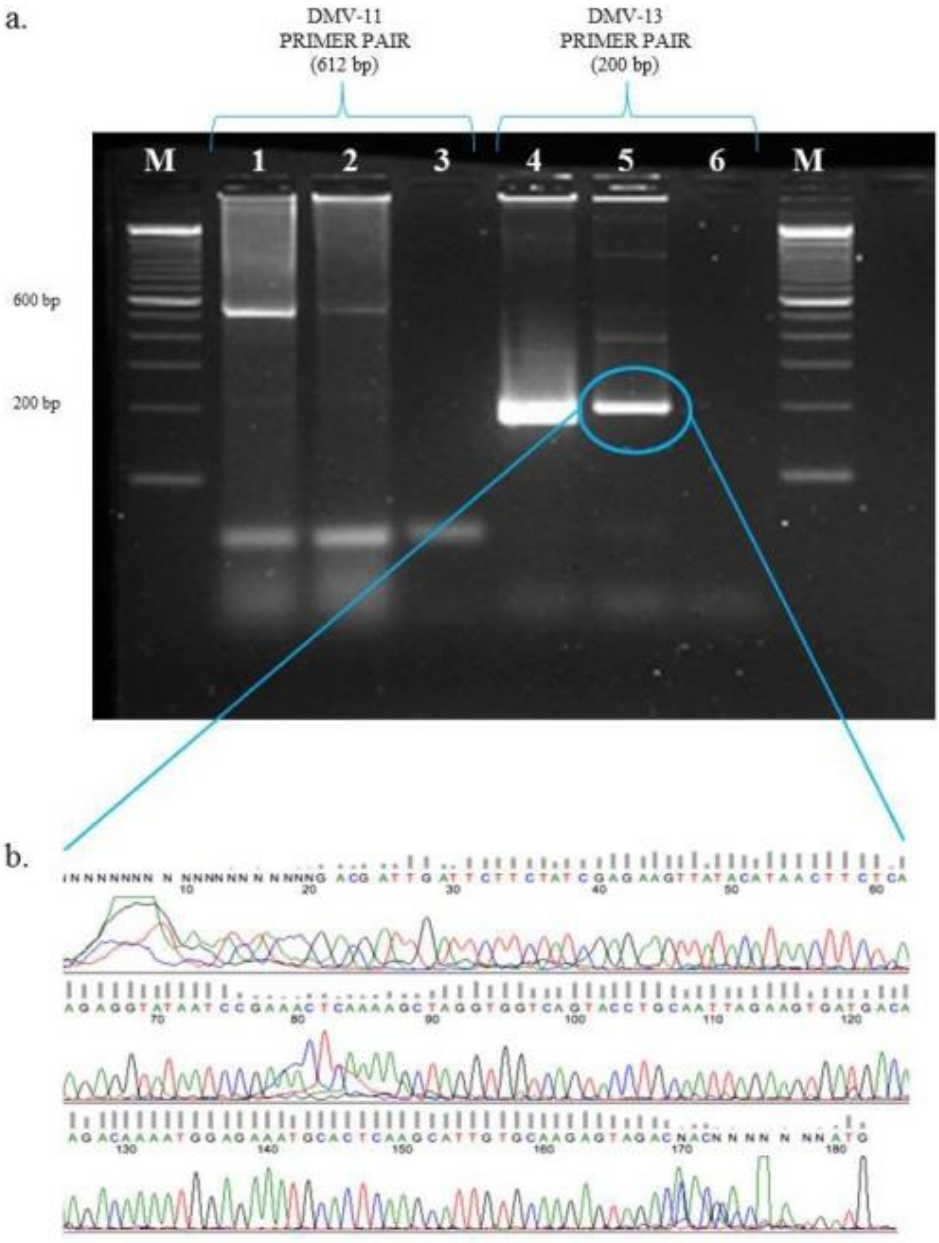


Figure 4.8. Agarose gel results of the second step of the nested PCR for CeMV using the DMV-13F and DMV-13R primer pairs on all the samples in Table 3.4.

RNA from all tissues was extracted using PureLink RNA Mini Kit (Ambion, Thermo Scientific).

M: DNA marker, 100 bp (DNA Ladder, Invitrogen); Lanes 1 and 2: *Stenella coeruleoalba*#1 (*SC#1*) lung and lymph node samples, respectively; Lanes 3, 4, 5 and 6: *Tursiops truncatus*#1 (*TT#1*) lung, brain, spleen and lymph node samples, respectively; Lanes 7, 8 and 9: *Phoca vitulina*#1 (*PV#1*) lung, brain, and lymph node samples, respectively; Lanes 10, 11 and 12: *Balaenoptera physalus*#1 (*BP#1*) lung, spleen and lymph node samples, respectively. Lanes 13, 14 and 15: *BP#2* lung, brain and spleen samples, respectively; Lanes 16, 17 and 18: *Physeter macrocephalus*#1 (*PM#1*) lung, brain and spleen samples, respectively; Lane 19, *PM#2* lung sample; Lanes 20 and 21: *PM#3* lung and kidney samples; Lane 22: PCR negative control.

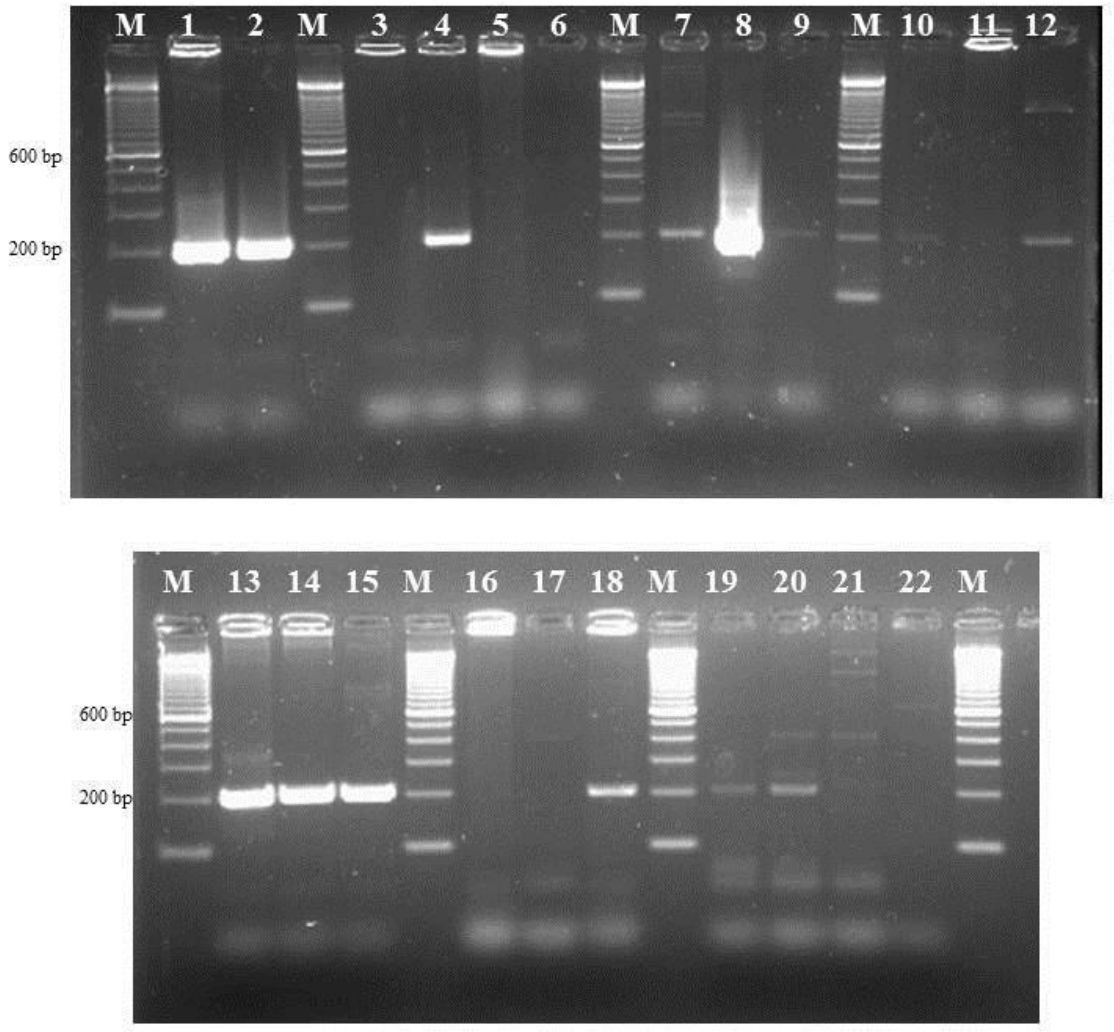


Figure 4.9. A) Agarose gel electrophoresis results for the conventional PCR protocol using primers DMV-N1 and DMV-P2 (Bellière et al., 2011; Mazzariol et al., 2016). The product size is 1358 bp. B and C) Agarose gel electrophoresis results for the second step of the nested RT-PCR protocol using primers DMV-13F and DMV-13R. The product size is 200 bp.

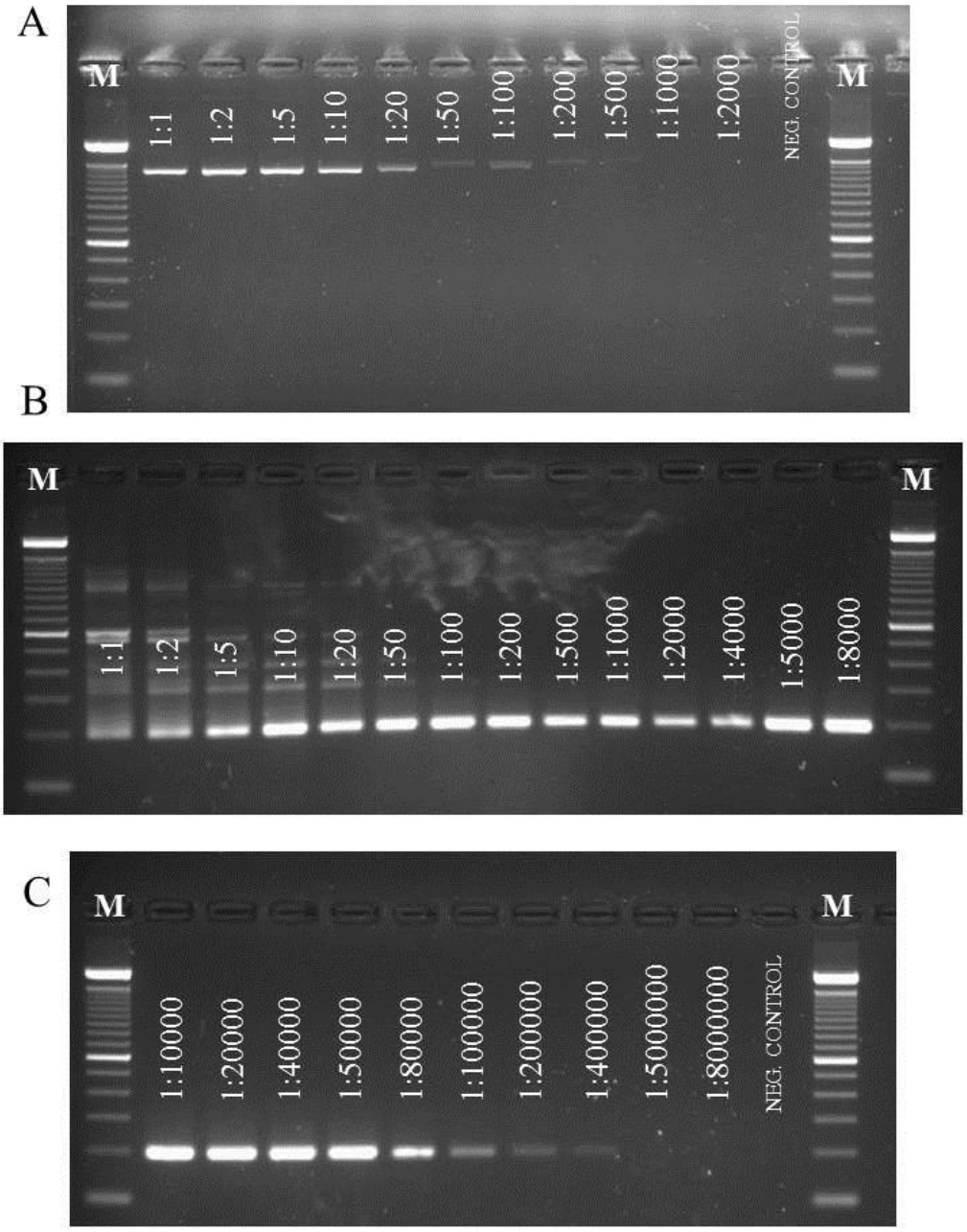
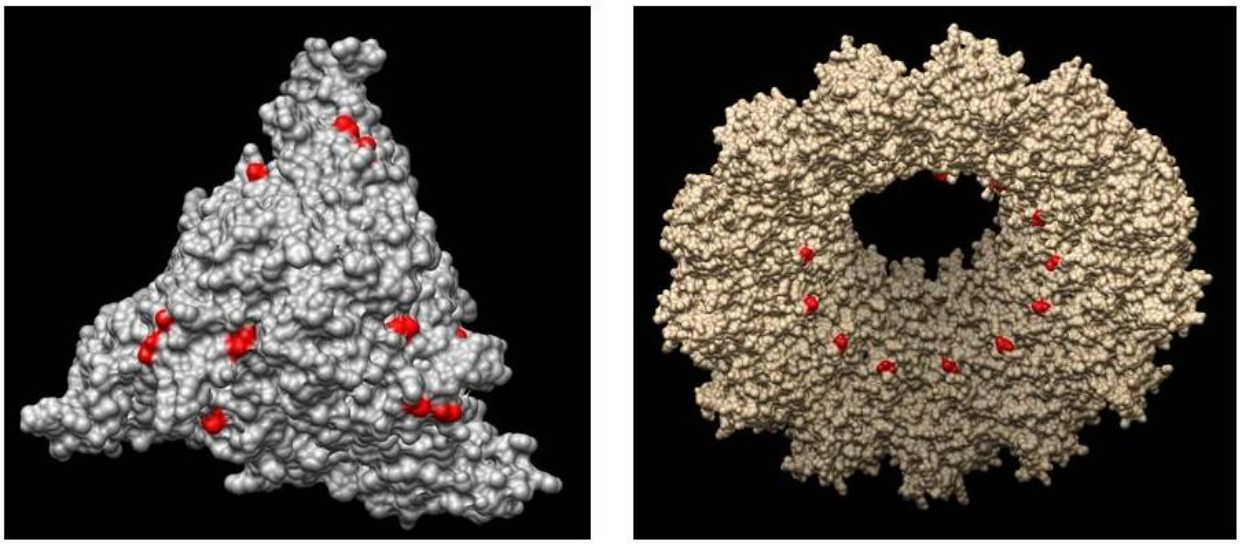


Figure 4.10. DMV homology modeling. Non-synonymous changes were exposed on the protein surface of the F protein (amino-acids 52) and the only amino-acid change modeled in the N protein (amino-acid 21) was located in the inner part of the capsid.



F polimer, top view
p.Asn52Ser

N polimer, bottom view
p.Ile21Thr

5. DISCUSSION

5.1 IMMUNE SYSTEM

Monoclonal antibodies which identify subpopulations of lymphoid organs' leucocytes are essential to investigate the role of these cells in the pathogenesis and regulation of diseases. Although cross-reactivity of antibodies between phylogenetically distant species has sometimes been observed (Jacobson et al., 1993; Jaber et al., 2013), a better understanding of immune function of cetaceans could be possible only using antibody validated for the species under study.

Cross-reaction of antibodies against human, bovine, mouse and ovine surface antigens such as HLA-DR, CD2, CD4, B cells and TCR has been reported on peripheral blood leukocytes of beluga whale (*Delphinapterus leucas*) and bottlenose dolphin (De Guise at al., 2002; Romano et al., 1992). Different monoclonal antibodies to lymphocyte surface antigens for cetaceans were also used in several research to study the subpopulations expression in lymph nodes (De Guise et al., 2002; Jaber et al., 2003): however these markers were considerate working on marine mammals just considering the positive immunolabelling of the cells and the similar pattern observed in the species of origin.

The cross-reactivity in striped dolphin (*Stenella coeruleoalba*) and bottlenose dolphin (*Tursiops truncatus*) of the monoclonal anti-human antibodies against respectively T and B cells surface and major histocompatibility complex were certified for the first time in this study. In fact, to confirm the specificity of anti-human antibodies used, the molecular weight of the surface proteins was evaluated and tested by Western Blotting, besides of the pattern of immunoreaction. Tested antibodies resulted to be suitable for immunohistochemical studies in formalin-fixed, paraffin-embedded tissues of striped dolphins and bottlenose dolphins. As expected the anti-human HLA-DR reacted strongly with cells of varying morphology that had characteristic of macrophages and dendritic-like cells of striped dolphin and bottlenose dolphin, whereas a huge number of lymphocytes were labelled by this antibody. The anti-CD3 marker positively stained T-cell zones, localized in the germinal center, in the mantle and marginal zone of lymph nodes sections, while anti-CD20 antibody stained B-cell in the para-cortical zone.

Furthermore, the selected commercial antibodies can be valuable tools also because they work on formalin-fixed, paraffin-embedded sample and not only on fresh or snap frozen tissues (Shirai et al., 1998; De Giuse et al., 2002).

Following the antibodies validation, albeit the small number of samples, a counting of leukocytes subsets was performed on immunostained tissues to understand the possible role of independent variables on lymphocytic cells subpopulations composition. Statistical analysis does not show statistically significant difference, except for the expression of anti-CD20 positive staining cells (B lymphocytes) in normal and hypoplastic lymph nodes, whose p-value was very close to the value considered indicative of a strong association. Analyzing the dolphins in the two groups we can realize that the 40% of the animals presenting hypoplastic lymphatic tissues are individuals with ongoing infections while the normal histology group include only animals apparently healthy.
Even if the number of animals included in the study is low due to the eligibility for storage characteristics of the tissue or the completeness of the medical history, we can speculate that, as already reported in the literature for some marine mammals' infections such as those from DMV (Van Bressem et al., 2014), the amount of lymphocytes to decrease as a result of immune depletion caused by the pathogens themselves. Morbillivirus infections have long been known to result in host immune suppression (Appel et al., 1982; Griffin et al., 1994). Recent studies suggest similar effects in naturally infected, wild bottlenose dolphins as part of capture-release studies for health assessment (Bossart et al., 2011). The author and his working group reported a significant decrease in mitogen-induced T cell proliferation along with an increase in lysozyme concentrations and a marginally significant increase in monocyte phagocytosis, along with a marginally significant decrease in the numbers of CD4+ T lymphocytes in bottlenose dolphin that had antibody titers $\geq$ 1:8 against morbillivirus, suggestive of previous exposure to the virus, compared to animals with lower or no titers. They found no effects on neutrophil phagocytosis. While the timing of the morbillivirus infection (active infection, chronic infection, resolved past infection) cannot be determined from titers alone, it is clear that there is an association between modulation of immune functions and previous exposure to morbillivirus infection in bottlenose dolphin, as observed in other species (Van Bressem et al., 2014). Therefore the data presented in literature seem to contrast those we encountered: a decrease of B lymphocytes instead of T lymphocytes. The researcher attention must be directed to the characterization of the T cell subsets in order to understand the subpopulation expression, possibly according to the timing of the infection. Therefore further studies are needed to understand the effective role of DMV as an immune suppression agent particularly in T cell populations validating and using CD4 and CD8 antibodies, marker of T helper cells and T cytotoxic cells respectively.

In addition to infectious agents, lymphoid depletion could be caused by environmental contaminants in cetaceans. Lymphoid organs hypoplasia is primarily associated with elevated

polybrominated diphenyl ether (PBDE) levels, while there is no correlation with the health status and nutritional state in bycaught animals, supporting the hypothesis of a contaminant-induced immune deficiency (Beineke et al., 2005). Additional toxicants, such as methylmercury and heavy metals are also suspected to negatively influence the immune system and increase disease susceptibility of harbor porpoises (Jepson et al., 1999; Siebert et al., 1999). The immunotoxic effect of several xenobiotics on cetacean blood leukocytes at concentrations equivalent to those observed in wildlife marine mammal populations has been verified in vitro. Noteworthy, DDT and non-coplanar PCB congeners inhibited spontaneous and mitogen-induced proliferation of beluga whale lymphoid cells, while coplanar (dioxin-like) PCB congeners and TCDD failed to modulate leukocyte function (De Guise et al., 1998). Further in vitro experiments confirmed their inhibitory effect on phagocytosis of neutrophils and monocytes of bottlenose dolphins and beluga whales. The dominating effect of non-coplanar PCB congeners is suggestive of a modulation of the leukocyte function in an aryl hydrocarbon receptor independent manner in these marine mammals (Levin et al., 2004). Similarly, mitogen-induced T and B cell proliferation is mainly modulated by non-coplanar PCB congeners, as investigated in killer whales, beluga whales, bottlenose dolphins and pilot whales, respectively (Mori et al., 2008).

In vitro experiments confirmed the negative influence of heavy metals on bottlenose dolphin leukocytes: mercury and cadmium decrease cell viability, phagocytosis and proliferation of these cells. In addition, they start lymphocyte apoptosis in concentrations equivalent to those reported in free-ranging cetaceans (Camara Pellisso et al., 2008). Similarly, in vitro exposure to mercury chloride and cadmium chloride decreases beluga whale splenocyte and thymocyte proliferation (De Guise et al., 1996).

Several studies on rodents indicate a potential immunotoxic effect of various marine xenobiotics, but metabolism and detoxification most likely differ between rodents and cetaceans (Levin et al., 2007; Mori et al., 2008). Further research in marine mammals has to be focused on evaluate if the biological activity of marine xenobiotics could cause a contaminant-induced hypocellularity with reduced numbers of CD4+ T helper cells or a reduced number of CD8+ cytotoxic T cells and an inhibited splenic humoral immune response such as lipophilic contaminants, as occurred in rodents (Beineke et al., 2010).

Recently, few research reported the use of lymphocytic antigen surface antibodies to characterized immunonocytologic features of a primary uterine T-cell lymphoma with multiple metastases in an adult female Atlantic spotted dolphin (*Stenella frontalis*) (Delgado et al., 2015), and to determine the immunophenotype of cellular inflammatory infiltrates in chronic cholangitis in 6 common

dolphins (*Delphinus delphi*) (Jaber et al., 2013). As reported before, the use of these antibodies was not validated by Western Blot.

On the contrary, the monoclonal anti-human antibodies validated for striped dolphin and bottlenose dolphin in this study could be useful as a IHC set to characterize the inflammatory infiltrate in different infection, such as *Toxoplasma gondi*. Indeed *Toxoplasma gondii* is a protozoan opportunistic pathogen for cetaceans and it has been recently reported in the brain of several striped dolphins with extensive meningoencephalitis lesions, all of which found stranded between 2007 and 2008 on the Ligurian Sea coast of Italy (Di Guardo et al., 2010), as well as in the brain of striped dolphins and bottlenose dolphins beached off the Italian Tuscany coast between 2006 and 2008 (Pretti et al., 2010). The study of the different lymphocytic subpopulation could be helpful to comprehend how different species presented in the Mediterranean basin, such as bottlenose dolphin and striped dolphin, respond to the pathogen depending on the environmental factor they are exposed and if the parasite stimulate a different inflammatory process.

5.2 DOLPHIN MORBILLIVIRUS

5.2.1 Nested PCR tecnique

RT-PCR is a valuable technique that is increasingly being used for the diagnosis of animal diseases caused by RNA viruses (Shin et al., 2004). In the present study, a more sensitive biomolecular technique based on conventional RT-PCR, the nested RT-PCR, has been implemented to detect DMV in badly preserved tissues from aquatic mammals.

An absolute prerequisite for the performance of RT-PCR is an efficient RNA extraction method. Currently, there are numerous protocols which can be used to isolate and purify RNA, although there are a few published studies comparing extraction methods for samples of animal origin (Deng et al., 2005). In our study, in which we compared two viral RNA extraction methods, PureLink RNA Mini Kit and TRIzol Reagent, we did not detect any relevant differences between the results obtained with the two aforementioned methods. However, RNAs extracted by the TRIzol Reagent constantly had the highest yield, while the extracts obtained by the PureLink RNA Mini Kit showed the highest A260/A280 ratio for almost all samples. In addition, after the first PCR step, the PCR products from samples extracted using the PureLink RNA Mini Kit showed a more intense signal following separation by agarose gel electrophoresis.

On the basis of these results, we can conclude that the two methods of RNA extraction are equally useful for the detection of CeMV in badly preserved aquatic mammal carcasses as well as in stranded whales.

For this reason, we focused our attention on the amplification method and we chose nested RT-PCR in order to detect CeMV even in poorly preserved samples.

The first set of primers selected in this study (DMV-11) was previously used to detect a 612 bp sequence of a conserved region of the DMV H gene (Mazzariol et al., 2016). The second set of primers (DMV-13) was designed on a short, specific, conserved region of the DMV H gene; this region was chosen to reduce the intraspecific variations among the different CeMV isolates hitherto characterized (Van Bressem et al., 2014).

We analyzed tissues from animals which had been already investigated in previous studies by molecular and/or immunohistochemical (IHC) analyses (Di Guardo et al., 2013; Mazzariol et al., 2013; Mazzariol et al., 2016), in order to test the specificity of the proposed nested RT-PCR method for CeMV detection. In these investigations, viral genome fragments were not always suitable for sequencing (Di Guardo et al., 2013), so an alternative approach was developed to obtain DNA fragments for sequencing studies (Mazzariol et al., 2013; Mazzariol et al., 2016). The results herein

obtained using nested RT-PCR are in agreement with those previously reported, except for the negativity observed in the BP#1 spleen. Notwithstanding what above, all the other tissues from BP#1 tested positive, so that we believe that two alternate hypotheses could be drawn in order to explain the aforementioned discrepancy. Indeed, while it seems plausible that such "negativity" may be due to an unsatisfactory preservation status of the spleen from BP#1, causing an intense viral RNA degradation, it might be additionally speculated that the previously reported positivity could have originated from RT-PCR nonspecific results, provided that the amplified genome fragment was not sequenced.

Furthermore, this technique allowed detection of DMV genome in animals not yet reported as giving positive results, both in small odontocetes, such as striped dolphin SC#1, where tissue degradation is very fast, and in stranded whales, such as sperm whales PM#1, PM#2 and PM#3, in which post mortem autolytic processes are increased by the size of the carcass, with subsequent rapid degradation of the RNA viral genome.

Due to the proven efficiency of the nested RT-PCR technique herein described, we believe this new method could be useful both to detect the presence of DMV infection in aquatic mammal tissues harbouring low viral loads and to easily sequence small amounts of viral genome, as also confirmed by previously described nested RT-PCR protocols (Shin et al., 2004; Sierra et al., 2014). Furthermore, this method could provide a valuable option for detecting DMV during the initial and final stages of the viral infection in susceptible host tissues, where morbilliviruses have been reported to be present in low amounts (Ludlow et al., 2015). In addition, the nested RT-PCR protocol herein reported may also turn out to be useful both in “retrospective” investigations on aquatic mammal specimens previously assessed as being Morbillivirus-negative (or whose Morbillivirus infection status has not been determined, as in the case of tissue bank specimens, for instance) and in “controversial” positive cases, as reported in recent mortality outbreaks occurring worldwide (Rubio-Guerri et al., 2013; Casalone et al., 2014; Stephens et al., 2014). In these recent events, indeed, the morphopathological lesions’ patterns did not always correspond to the “classical” disease phenotype described in the literature (i.e. subacute-to-chronic broncho/bronchiolo-interstitial pneumonia with “Warthin-Finkeldey type” multinucleate syncytia, along with subacute-to-chronic, non-suppurative meningoencephalitis). Moreover, morbilliviral infection was not always detected by means of the “canonical” laboratory diagnosis tests, such as IHC and conventional RT-PCR analyses.

In conclusion, the nested RT-PCR protocol presented may be considered a sensitive and rather cheap biomolecular technique, which is able to identify DMV-positive samples from aquatic mammals also when poorly preserved.

Despite these promising results, further studies should be conducted in order to increase the efficiency of this nested PCR technique, as well as in order to understand if the same method could be also useful to detect CeMV through the amplification of other evolutionarily conserved viral genes, such as the P and the nucleocapsid protein (N) genes.

5.2.2 DMV in fin whales

The results of direct (IHC and biomolecular) and indirect (serologic) testing provide evidence of DMV infection or exposure in 5 (55%) of 9 fin whales that were found stranded along the Mediterranean Coast during 2011–2013. These 5 infected whales correspond to 21.7% of the 23 whales stranded along the Italian coastline during 2006–2014. The other 4 examined whales showed no evidence of morbillivirus infection. The range of DMV-susceptible host species has progressively expanded (Van Bressem et al., 2014), as highlighted by the recent report of DMV infection in a captive harbour seal (*Phoca vitulina*) during the 2011 outbreak (Mazzariol et al., 2013). This expansion, combined with spread of DMV through the transplacental route, resulting in virus colonization of the thymus in fetuses, could represent DMV survival strategies among cetacean populations. In addition, our data argue in favor of an epidemic cluster of fatal DMV among the Mediterranean fin whales population, even though, on the basis of the amino acid sequence of the SLAM/CD150 viral receptor, this species is not included among those susceptible to DMV epidemics (Van Bressem et al., 2014; Ohishi et al., 2010).

Although the single amino acid substitution, p.Ala451Thr, did not cause substantial variations in the structure of H antigen, the effect of the variation on protein functions is unclear. Recent studies showed that similar amino acid changes could affect virulence and infectivity of different canine distemper virus (family *Paramyxoviridae*, genus *Morbillivirus*) strains, but such changes are often neutralized by compensatory mutations that preserve the biologic activity of H protein (Satter et al., 2014). Furthermore, despite the high sequence homology observed between N, P, and H genes of the DMV strain identified in the newborn fin whale and in the isolates recovered from DMV-affected cetaceans during the 1990–1992 and the 2006–2007 epidemics (GenBank accession no. AJ608288), we cannot exclude that more prominent differences occurred in virus genes encoding for both structural and nonstructural proteins responsible for virulence and pathogenicity (e.g., P/V/C and fusion genes) (Van Bressem et al., 2014); the simultaneous occurrence of primary structure differences, if any, in the SLAM/CD150 receptor should also be taken into account (Ohishi et al., 2010). In conclusion, although further studies are needed to elucidate the complex virus–host interaction dynamics and the putative influence exerted by environmental factors, DMV

should be regarded as one of the major threats for the conservation of fin whales within the Mediterranean Sea.

5.2.3 DMV in sperm whale

The positive sequences obtained from the 4 sperm whales' tissues were analyzed by means of an hoc computer program and the H consensus fragment obtained from all the positive samples showed 100% sequence homology with the corresponding DMV genome sequence (GenBank Acc. No. AJ608288). Simultaneous immunohistochemical evidence of DMV nucleoprotein (N) antigen was additionally observed in macrophages and follicular dendritic-like cells from the spleen's white pulp in the youngest female *(#PM1*), as well as in monocytes circulating within splenic blood vessels from the same animal.

Despite no "classical" DMV-related pathological changes being observed during post mortem investigations3,4, viral circulation was strongly suspected in at least in 2 out of the 3 DMV-infected sperm whales based on IHC (*#PM1*) and biomolecular *(#PM1*, *#PM2* and *#PM3*) findings. In this respect, the consistent morbilliviral antigen's immunolabeling both in circulating monocytes and in splenic follicular-like dendritic cells, support the hypothesis that DMV infection was in an early developmental stage (De Swart et al., 2007). Experimental studies conducted on similar morbilliviruses show that during this period, even if no severe clinical signs may be expected, a "general discomfort condition", secondary to a viremic circulation could be suspected (De Vries et al., 2012; Stein et al., 2008).

While DMV infection has been often associated to mass mortalities during epidemic outbreaks (De Swart et al., 2007), it has been seldom described in single mass stranding events, not only in sperm whales but, more in general, in cetaceans. In this respect, while sperm whales' susceptibility to Cetacean Morbillivirus (CeMV) has been recently documented, alongside with the likely occurring materno-fetal transfer of the virus (West et al., 2015), the biomolecular evidence of DMV infection obtained in the fetal sperm whale herein investigated strongly supports the hypothesis of a transplacentally acquired infection in this animal.

Although no clear-cut evidence exists that DMV was the primary cause of the sperm whales' mass stranding reported herein, a huge body of scientific literature is available to support the primary pathogenicity of Morbillivirus genus members for their mammalian hosts, including aquatic mammals (Van Bressem et al., 2014). Despite previous studies suggest chemical pollutants as one of the relevant causative factor in determining stranding and subsequently death of the 3 stranded cetaceans under study (Squadrone et al., 2015), the present investigation underline a possible role of

DMV infection which could have determined their entrance northward into the Adriatic Sea, which is known to be a "cul-de-sac" for this species (Mazzariol et al., 2011).

In conclusion, while it is easier to support the direct involvement of CeMV “clade” members such as DMV to the several epidemic outbreaks occurred in the last 25-30 years among free-ranging cetaceans worldwide (Van Bressem et al., 2014), their role, as well as other factors, in single mass stranding events cannot be defined precisely since biological, ecological and environmental co-factors should be also investigated by using a multidisciplinary study effort (Mazzariol and Di Guardo, 2016).

6. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The recent discoveries of several new morbilli-related viruses in bats (Drexler et al., 2012), as well a potentially novel feline morbillivirus representing a basal divergence in the genus (Woo et al., 2012), are likely to lead to understanding of the evolution of morbilliviruses. Morvilliviruses are a growing concern because of their ability to infect multiple species as recently reported for canine distemper virus infected Amour tiger (*Panthera tigris altaica*) and lion (*Panthera leo*) populations (Seimon et al., 2013; Viana et al., 2015) probably caused by a spill-over of CDV from domestic dog associated with severe decline in wild carnivores worldwide and mass dog vaccination.

The single mutations herein described in the DMV genomic sequence and the recent results of diagnostic surveillance performed by public veterinary Institutions involved in the Italian Stranding networks, which confirm that 19% of cetaceans found dead along the Italian coastline were molecularly positive for DMV in 2015 without any evidences of ongoing epidemic event (Italian Diagnostic report on stranded cetaceans - 2015), argue in favor of an endemic circulation of DMV among Mediterranean cetaceans resulting in increased infectious pressure.

DMV epidemiology depends upon several factors, such as its virulence and herd immunity population density (Beineke et al. 2015). The viral strains that were responsible for the strandings herein described exhibit a marked genetic relatedness with those which have determined large mass mortalities in early nineties and in 2006 (Van Bressem et al., 2014). This observation, supports simultaneously a prolonged DMV circulation in the Western Mediterranean as well as the hypothesis of an inadequate level of anti-viral immunity in cetaceans inhabiting this area.

During endemic circulation of virus belonging to this genus, periodic and self-limiting disease outbreaks may occur, due to a decreased level of the population's antiviral immunity, as occurred in 2011 (Di Guardo et al., 2014) and 2013 (Casalone et al., 2014).

Of great interest are the documented cases of DMV infection in the fin whale (*Balaenoptera physalus*) newborn (Mazzariol et al., 2016) and in the sperm whale (*Physeter macrocephalus*) *foetus*, which confirms the possibility of vertical transmission. The sperm whale mother was also DMV-infected, was part of a DMV-infected, female sperm whales' pod. To our knowledge, this should be one of the few documented cases of DMV involvement in a cetacean mass stranding and the only reported example of DMV involvement in a sperm whales' mass stranding worldwide.

In these news species vertical transmission is hypothesized as the likely route of entry as well as the possible survival strategy of the virus. (West et al., 2015; Mazzariol et al., 2016). The involvement

of newborns, young and pregnant females, could represent a serious menace for the conservation of these already threatened species. This potential "infection strategy" could provide the virus with a stronger pathogenicity, which could be further enhanced by the immune suppression para-physiologically such as occurring during pregnancy (Sykes et al., 2012). In this respect, the recently described biomolecular and immunohistochemical evidence of DMV in the thymus of the newborn fin whale (Mazzariol et al., 2016) provides additional support to the aforementioned hypothesis, given that an immunotolerance-like condition against the virus presumably developed in this whale (Weissman, 2016).

The changing infection process of DMV toward its "traditional" and its "new" hosts could be explained both by a population's immunity enhancement resulting from prolonged DMV exposure (Profeta et al., 2015) and by the evolving lesions' pattern.

While the viral "infectious pressure" may be reasonably expected to increase under similar conditions, a simultaneous hosts' immune response impairment, either age or pregnancy (having many of the recent cases of DMV infection been diagnosed in newborns, calves or pregnant females) or otherwise pollutant-related, may facilitate cross-species infection. In fact, it is unknown whether the high tissue levels of immunotoxic chemical substances commonly found in Mediterranean cetaceans (Panti et al., 2011) are responsible for the lack of protective immunity against one or more of the CeMV strains circulating for years in this region (Di Guardo et al., 2013).

The density of the recipient host population is important in the transmission of any transferred virus (Parrish et al., 2008), but in the last years, monitoring programs dedicated to estimates cetaceans abundance in the Mediterranean basin denounced a decreased presence of striped dolphins (*Stenella coeruleoalba*) in the Thyrrenian Sea (Panigada et al., 2011). The lower density of striped dolphins and bottlenose dolphins (*Tursiops truncatus*), considered the classic hosts for DMV (Sato et al., 2012), could have play a role in the cross-species infection, along with other potential factors as an intermediate and amplifier hosts with the critical role of bringing DMV in close contact with alternative hosts, as well as factors which change the geographical distribution of the species or decrease the behavioral separation In fact, the rate and intensity of contact may be relevant as well as the evolutionary relatedness (Parrish et al., 2008). These new species, similarly to wild carnivores for CDV, could act as reservoir being a possible cause of spillback events involving naive population of classic hosts and supporting the presence and circulation of the virus in this limited basin (Beineke et al, 2015; Mazzariol et al., 2016).

Further research on the challenging topic of morbilliviral infections in cetaceans and, more in general, in aquatic mammals is warranted. To this aim, identification of the SLAM/CD150 cell

receptor in several cetacean species (Ohishi et al., 2010; Shimizu et al., 2013) represents a major step in our understanding of the pathogenesis of DMV infection, especially with regard to susceptibility and transmission to non-classical hosts, such as pinnipeds and fin whales (Mazzariol et al., 2013; Mazzariol et al., 2016). Further studies should confirm whether the SLAM/CD150 cell receptor is indeed the primary immune receptor for CeMV, as is the case for other studied morbilliviruses, and should look for the nectin4 epithelial cell receptor and other cell receptors in cetaceans. It will be interesting also quantify the expression of SLAM/CD150 to understand if in the animal affected by DMV the virus causes an over-expression of its receptor.

Further studies are also warranted to delineate the host responses to CeMV strains and lineages, and the factors that determine the outcome of infection in cetaceans. Mathematical models should be developed to examine the long-term dynamic consequences of the epidemics on cetaceans populations and to predict the risk of epidemics (Van Bressem et al., 2014).

Further analyzes will be also aimed at analyzing the relationship between CD4+ and CD8+ lymphocytic cells in order to understand the effect of the DMV, important immunosuppressant agent, on T cell populations. Finally, it could be interesting compare the data obtained on the immune system with those obtained from the same samples as regards the search for environmental pollutants.

The study of the different lymphocytic subpopulation expression in DMV affected animals using monoclonal validated antibodies, together with an integrative approach that includes epidemiological parameters, life history of the affected species and environmental parameters should provide a better and more complete picture of the ecology and evolution of DMV, and, more in general, of CeMV.

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9. SCIENTIFIC CONTRIBUTIONS TO CONGRESSES

Oral presentation: Capture myopathy in 16 cetaceans stranded along the Italian coastline between 2008 and 2014. *29th European Cetacean Society Conference*, 23-25 Marzo 2015;St. Julians (Malta).

Molecular detection of dolphin morbillivirus: a new fast and sensitive detection system based on nested RT-PCR. *30th European Cetacean Society Conference,* 14-16 Marzo 2016; Funchal (Madeira).

The brain of large whales. *21th Biennial Conference on the Biology of Marine Mammals*. 11-18 Dicembre 2015; San Francisco (CA).

Cutaneous macroscopic and microscopic lesions caused by *Pennella sp*., *Crassicauda sp*., *Phyllobothrium delphini* in Cetaceans. *28th Annual Congress of the European Society and College of Veterinary Dermatology*, 24-26 Settembre 2015; Cracovia (Polonia).

Cross-reactivity evaluation of commercial antibodies against leukocyte sub-populations in striped dolphins (*Stenella coeruleoalba*). *V Convegno Nazionale della Ricerca nei Parchi – L'Uomo per gli Animali*, 10-12 Ottobre 2014; Bussolengo (Verona).

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