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# NEUROPATHOLOGIC FINDINGS IN CETACEANS STRANDED IN ITALY (2002–14)

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ABSTRACT: We summarized the neuropathologic findings in 60 cetaceans stranded along the Italian coastline from 2002 to 2014. The following neuropathologic changes were detected in 45% (27/60) of animals: nonsuppurative meningo-encephalitides (30%, 18/60), nonspecific lesions (12%, 7/60), suppurative encephalitis (2%, 1/60), and neoplasm (2%, 1/60). No histologic lesions were found in 47% (28/60) of the specimens. Five (8%, 5/60) samples were unsuitable for analysis. Analysis with PCR detected *Brucella* spp., morbillivirus, and *Toxoplasma gondii* infection in one, six, and seven individuals, respectively. Immunohistochemical analysis confirmed positivity for morbillivirus and for *T. gondii* infection in three cases each. No evidence of the scrapie-associated prion protein PrPSc was detected. Our findings underscore the importance of an adequate surveillance system for monitoring aquatic mammal pathologies and for protecting both animal and human health.

*Key words:* *Brucella* spp., marine mammals, morbillivirus, neuropathology, stranding, *Toxoplasma gondii*.

## INTRODUCTION

Determining the cause of strandings is of paramount relevance for the assessment of the health status of free-ranging cetacean populations. Marine mammal strandings provide a valuable source of information about the animals for environmental analyses (Bossart et al. 2011). Several pathogens can infect cetaceans, among which *Brucella* spp. and *Toxoplasma gondii* are recognized zoonotic agents. Other pathogens that can cause episodes of mass mortality include morbilliviruses, which have been responsible for pinniped and cetacean die-offs worldwide in the past 30 yr (Di Guardo 2012; Casalone et al. 2014; Van Bressem et al. 2014).

*Brucella* spp. infections were first described in pinnipeds and cetaceans in 1994 (Ewalt et al. 1994; Ross et al. 1994), and they have since been reported in many countries (Dawson 2005; Tachibana et al. 2006; Ohishi et al. 2007). Since 2007, isolates of *Brucella* spp. from marine mammals have been further classified by molecular methods into two species, *Brucella ceti* and *Brucella pinnipedialis*, species that infect cetaceans and pinnipeds, respectively (Foster et al. 2007). *Brucella* spp. were identified in 2009 in a dolphin that had stranded on the Mediterranean Catalonian coast (Isidoro-Ayza et al. 2014); other cases have been described in the same area and along the coast of Italy (Alba et al. 2013; Grattarola et al. 2016).

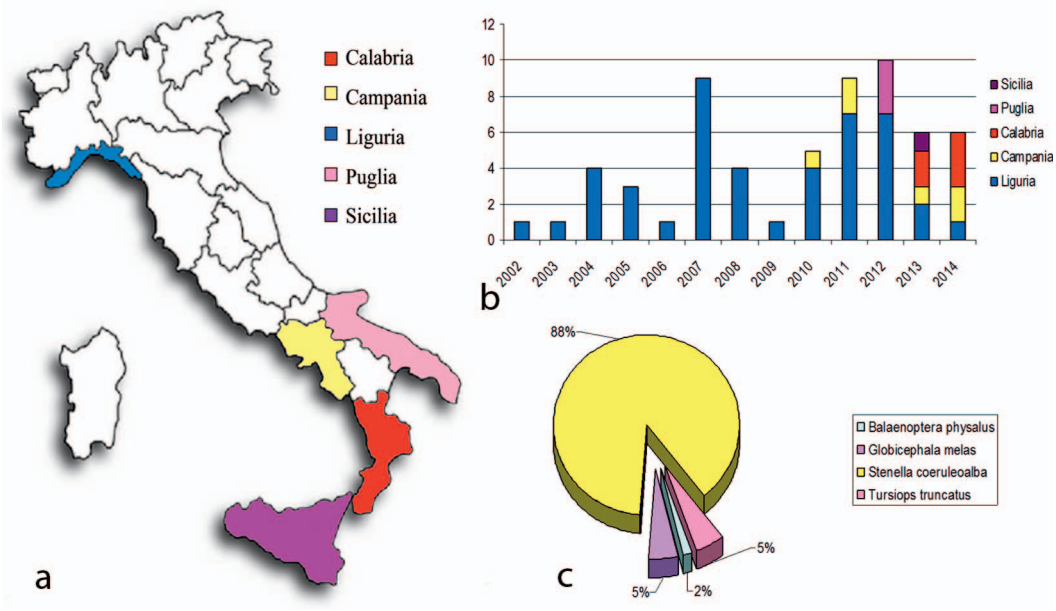


FIGURE 1. Geographical distribution of cetacean strandings (2002–14) in Italy, along with the stranding rates and the species of cetaceans involved. (a) Map of Italy showing, in different colors, the regions where the cetaceans stranded (2002–14). (b) Annual distribution of the strandings by region. (c) Percentage of the different cetacean species involved.

Morbilliviruses are lethal viruses that cause severe lesions in the brain, lung, epithelial, and lymphoid tissues of marine mammals. Since 1988, at least four species have been classified as being pathogenic for aquatic mammals: cetacean morbillivirus (CeMV), phocine distemper virus, canine distemper virus (CDV), and monk seal morbillivirus. Cetacean morbillivirus includes three main strains—porpoise morbillivirus, dolphin morbillivirus (DMV), and pilot whale morbillivirus—along with the newly characterized CeMV clade members from Hawaii and the southern hemisphere (Di Guardo et al. 2005; Van Bressem et al. 2014; Jacob et al. 2016).

*Toxoplasma gondii* is a protozoan implicated as a cause of systemic disease in several marine mammal species. It is believed to be an opportunistic pathogen for aquatic mammals (Migaki et al. 1990; Miller et al. 2002), as exemplified by the DMV epidemic that involved Mediterranean striped dolphins (*Stenella coeruleoalba*) from 1990 to 1992 (Domingo et al. 1992). Sporadic cases of fatal toxoplasmosis have also been reported in several cetacean and pinniped species, with or without immunosuppression due to concurrent morbillivirus infection (Miller et al. 2008; Di Guardo et al. 2010).

All these pathogens can affect the central nervous system, resulting in fatal acute or chronic meningoencephalitis. Furthermore, a prion-like disease was recently reported for the first time in a marine mammal, indicating that this type of neurodegenerative disorder can affect aquatic animal species (Di Guardo et al. 2012).

Our aim was to describe and discuss the neuropathologic findings detected in the brain tissue of cetaceans stranded along the Italian coastline from 2002 to 2014 and submitted to the Neuropathology Laboratory of the National Reference Centre for Diagnostic Investigations on Stranded Marine Mammals (C.Re.Di.Ma.–Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Torino, Italy).

## MATERIALS AND METHODS

Between January 2002 and December 2014, we collected 60 brains in total from cetaceans found

stranded along the Italian coastline (Fig. 1a). They were examined at the Neuropathology Laboratory of C.Re.Di.Ma. We did not include cetaceans involved in the unusual mortality event that occurred along the Tyrrhenian Sea coast of Italy during the first 3 mo of 2013 (Casalone et al. 2014).

The 60 stranded cetaceans were confirmed as 53 striped dolphins (36 males and 17 females), two male and one female common bottlenose dolphin (*Tursiops truncatus*), three female pilot whales (*Globicephala melas*), and one male fin whale (*Balaenoptera physalus*; Fig. 1b). The annual distribution and geographic origin of the animals is shown in Figure 1c. The brain of each animal was divided in two parts by a paramedian cut; the larger portion was fixed in 10% buffered formaldehyde solution for histology and immunohistochemistry (IHC), and the smaller portion was frozen at –20 C for biomolecular analyses.

### Histology

Coronal sections of formalin-fixed brain regions (telencephalon, diencephalon, mesencephalon, pons, cerebellum, medulla) were trimmed. The tissues were routinely processed, embedded in paraffin, and 4-µm-thick tissue sections were cut for histopathologic and IHC analyses. The sections for histopathology were stained with H&E, and the histologic lesions were categorized.

### PCR analysis

Frozen brain tissue samples were submitted to PCR for the detection of *Brucella* spp., *T. gondii*, and DMV. To extract DNA and RNA, tissue samples (30–50 mg) were physically disrupted on a TissueLyser II homogenizer (Qiagen, Hilden, Germany) by high-speed shaking in plastic tubes with stainless steel beads (5 mm in diameter). Genomic DNA and total RNA were then extracted from the disrupted tissues with an AllPrep DNA/RNA Mini kit (Qiagen) according to the manufacturer's instructions.

The reverse transcriptase-PCR for the diagnosis of morbillivirus infection is targeted to a highly conserved region of the nucleoprotein (*N*) gene belonging to CDV and DMV (Verna et al. 2017). We used a nested PCR for ribosomal DNA locus to test for *T. gondii* (Vitale et al. 2013). We detected *Brucella* spp. by means of a hemi-nested PCR targeting an outer membrane protein gene of *Brucella abortus* (Baily et al. 1992). The PCR products were analyzed by electrophoresis on 2% agarose gel containing GelRed (Biotium, Fremont, California, USA) in comparison with molecular weight markers and subsequently photographed on a Gel-Doc UV transilluminator system (Bio-Rad, Hercules, California, USA).

### Immunohistochemistry

Brain samples from animals showing neuropathologic changes or that were positive by PCR were submitted to IHC analyses for the detection of *T. gondii* and DMV. We performed IHC for *T. gondii* as reported previously (Di Guardo et al. 2010). For morbillivirus antigen detection, sections were deparaffinized, rehydrated, and warmed in a water bath at 90–95 C for 20 min in citrate buffer (pH 6.1). Endogenous peroxidase activity was quenched with hydrogen peroxide (3% in methanol) for 20 min at room temperature. The primary commercially available (VMRD, Pullman, Washington, USA) anti-CDV nucleoprotein mouse monoclonal antibody (MAb), diluted 1:500 in Tris-buffered saline with Tween (TBST), was then applied for 2 h at room temperature. Subsequent antibody detection used an anti-mouse secondary antibody (diluted 1:200 in TBST) for 30 min at room temperature, followed by the avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, California, USA). Immunoreactivity was visualized using 3-3′-diaminobenzidine as the chromogen, and the sections were counterstained with Mayer's hematoxylin.

An in-depth IHC investigation, aimed at assessing the occurrence of the "disease-associated isoform" of the cellular prion protein (scrapie associated prion protein [PrPSc]), was done on all brain tissue samples. For this purpose, sections of the medulla, cerebellum, and frontal cortex from each brain were selected to detect the presence of PrPSc by using the 6H4 primary MAb (Prionics, Schlieren, Switzerland) diluted 1:1,000 in TBST, as described previously (Iulini et al. 2012).

## RESULTS

Histologic lesions were detected in 45% (27/60) of brain samples; no neuropathologic changes were observed in 47% (28/60) of animals. The brains of 8% (5/60) of cetaceans resulted in advanced postmortem autolysis by neuropathologic examination and therefore were unsuitable for a diagnosis. Nonsuppurative meningo-encephalitides were observed in 18 animals, in which mild-to-severe lesions were present. Lymphocytes, plasma cells, and macrophages were the predominant cell types and a few polymorphonuclear leukocytes were visible in seven cases. The microscopic

hallmarks were vasculitis, perivascular cuffing, and meningitis. In the cases with severe lesions, distension of the perivascular space by an inflammatory exudate was observed, with the surrounding nervous tissue showing diffuse gliosis or isolated glial nodules in the areas immediately adjacent to the vascular lesions. In the cerebral cortex, neuronal necrosis, satellitosis, and neuronophagia were also seen (Fig. 2a). Basophilic spherical structures, isolated or clustered, variably scattered or arranged in chains and morphologically consistent with protozoan cysts, were found in the brain parenchyma of three animals (Fig. 2b).

Seven samples showed nonspecific lesions characterized by gliosis, microgliosis, or both; neuronal degeneration; and neuronophagia in the gray matter. Focal mononuclear cell infiltrates were also apparent in vessel walls in two animals, and a small malacic area in the cerebellum was visible in another animal. Severe suppurative meningitis and choroid plexitis were detected in one animal. Polymorphonuclear perivascular cuffs at the level of periventricular areas and an abscess at the level of thalamus were also seen (Fig. 2c).

A tumor identified as a glioblastoma or a high-grade astrocytoma was detected in one animal. But because the entire brain was not available, it was impossible to topographically define the lesion site. Histopathologic examination showed a noncapsulated and infiltrating, space-occupying mass characterized by poorly differentiated and polymorphic cells, ranging from round to fusiform, with undefined cytoplasmic borders and prominent nucleoli; multinucleated giant cells were occasionally seen. Vascular proliferation, hemorrhagic foci, and neoplastic cells pseudopalisading around multiple areas of necrosis were also observed (Fig. 2d).

We performed PCR on brain tissue samples from 45 animals; the frozen portion from the remaining 15 animals was not available. Samples were positive for DMV (Fig. 3) and *T. gondii* in 13% (6/45) and 15% (7/45) of specimens, respectively. Almost all of the animals were Mediterranean striped dolphins (Table 1), except for a common bottlenose dolphin (case 9) and a pilot whale (case 11). One animal tested positive for *Brucella* spp. (Garofolo et al. 2014).

Using IHC, we analyzed samples from 30 animals with brain lesions or that tested positive by PCR. Morbillivirus IHC was positive in three cases, one of which was unsuitable (case 12), probably with a subclinical morbilliviral infection (Van Bressem et al. 2014); the other two exhibited mildly severe lesions (cases 5 and 7). No neuropathologic changes were noted in the third animal (case 11), in which a subclinical morbilliviral infection may have been present (Van Bressem et al. 2014). The presence of *T. gondii* cysts was confirmed in the brain tissue of three striped dolphins (Fig. 2e), two of which (cases 5 and 12) also tested positive for morbillivirus (Fig. 2f). Neuropathologic examination of case 5 showed meningitis, perivascular cuffing, gliosis, and neuronophagia presenting with different degrees of lesion severity. Cytoplasmic inclusions, nuclear inclusions, syncytial cells, and malacia were absent, whereas protozoan cysts were present. The animal also showed pulmonary lesions with the presence of syncytial cells (unpublished data), suggesting that this case could be classified as a chronic, systemic form of DMV infection (Van Bressem et al. 2014). Interestingly, although the sample from case 12 seemed unsuitable, as only a few autolyzed brain sections were available, protozoan cysts were detected even in the absence of histologic lesions. All the samples examined for PrPSc IHC were negative.

## DISCUSSION

Postmortem examination investigating the cause of death in stranded cetaceans is an extensive process. Due to the postmortem decomposition of cetacean carcasses, however, this is often not possible, with the result that much valuable data may be lost and disease cases underreported. Moreover, infectious diseases found in stranded cetaceans frequently have a complex pathogenesis involving other cofactors such as persistent

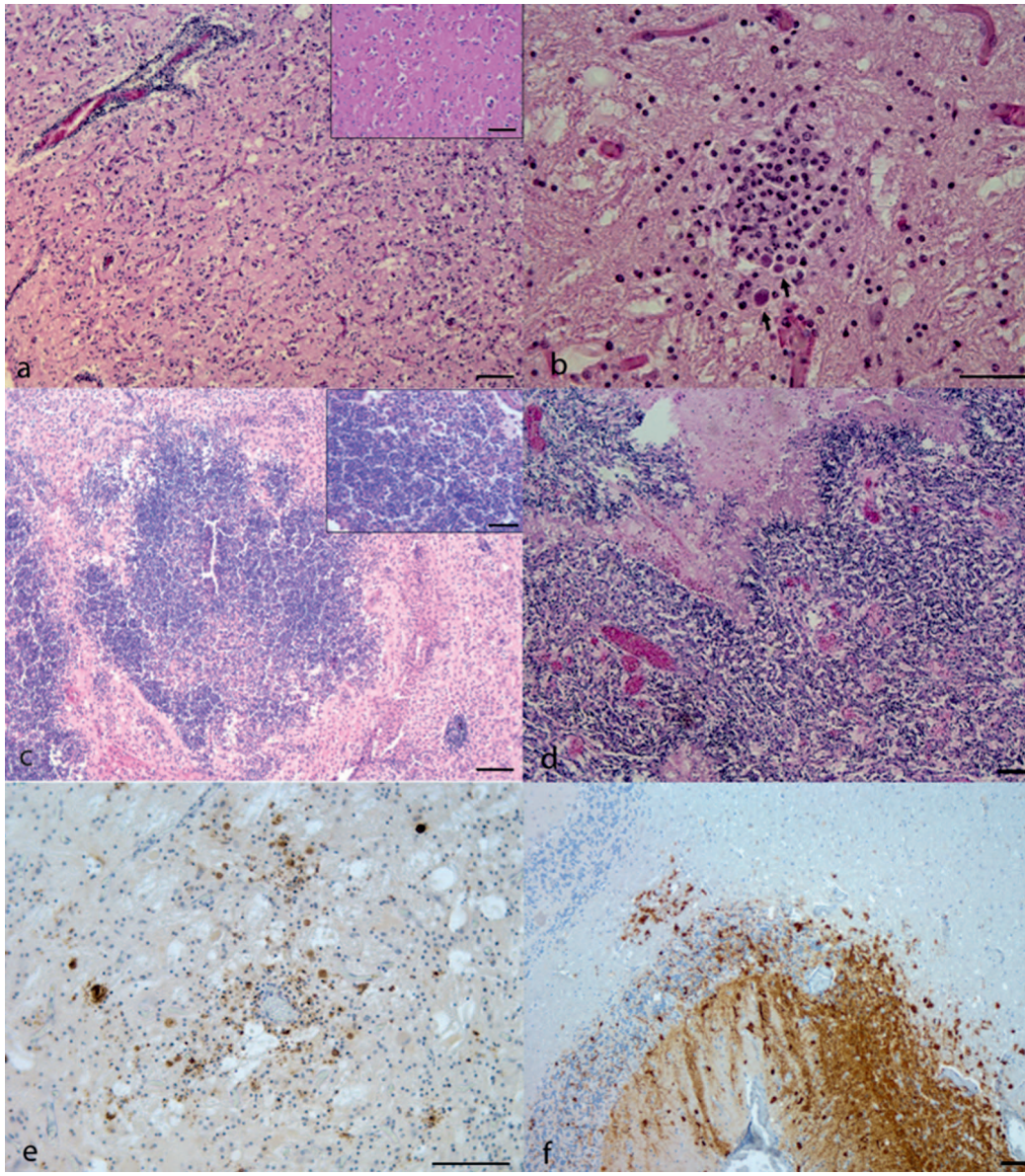


FIGURE 2. Neuropathologic lesions observed in cetaceans that stranded in Italy (2002–14). (a) Morbillivirus infection, cerebral cortex, *Stenella coeruleoalba*. Edema in the gray matter associated with mononuclear perivascular cuffs, vascular proliferation, gliosis, and neuronal necrosis. H&E stain. Bar=100 µm. Inset: gliosis and neuronal necrosis. H&E. Bar=100 µm. (b) Toxoplasmosis, thalamus, *S. coeruleoalba*. Glial cells mixed with inflammatory cell infiltrate, including macrophages, lymphocytes, neutrophils, and protozoal cysts (arrows). H&E stain. Bar=50 µm. (c) Abscess, thalamus, *S. coeruleoalba*. Unorganized abscess with a necrotic center, including degenerated neutrophils and surrounded by macrophages infiltrating the adjacent parenchyma. H&E stain. Bar=100 µm. Inset shows neutrophil granulocytes. H&E stain. Bar=50 µm. (d) Glioblastoma, brain, *S. coeruleoalba*. Microvascular proliferation and areas of necrosis bordered by neoplastic pseudopalisading cells. H&E stain. Bar=100 µm. (e) Toxoplasmosis, brainstem, *S. coeruleoalba*. Positive immunohistochemical labeling of tachyzoites and parasitic cysts scattered throughout the parenchyma. Bar=100 µm. (f) Morbillivirus infection, cerebellum, *S. coeruleoalba*. Positive immunohistochemical labeling of Purkinje cells, molecular and granular layers, and inflammatory cells. Bar=100 µm.

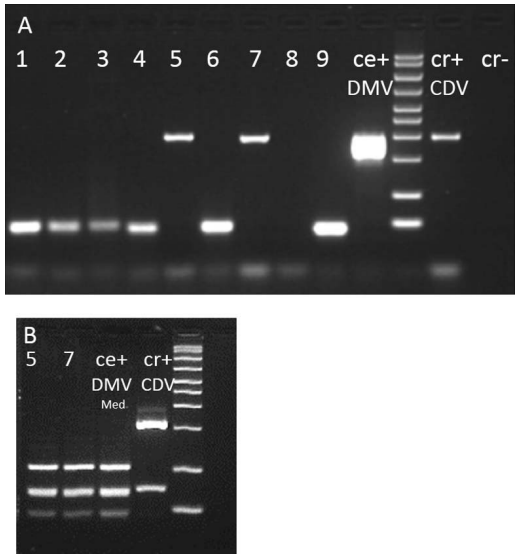


FIGURE 3. Dolphin morbillivirus (DMV) genomic RNA was detected in cetacean brain tissue specimens by using a reverse transcriptase-PCR targeting a 287-base pair (bp) segment of the *N* gene and restriction analysis of PCR products with *MseI* enzyme was then applied to confirm specificity of positive reactions (Verna et al. 2017; 50–2,000 bp Ladder, Bio-Rad). (A) PCR results are reported for nine of 45 tissue samples collected from cetaceans stranded along the Italian coastline from 2002 to 2014 and submitted to DMV detection. Lanes 5 and 7: positive reactions with amplicons at the expected molecular weight (287 bp). The PCR control panel was set up with a DMV-positive tissue (lane DMVce+) and a canine distemper virus (CDV) RNA (lane CDVcr+) for checking efficiency, respectively, of the genomic RNA extraction step and the amplification reagent mix, in addition to a blank sample (lane cr–) to monitoring operational effectiveness. (B) Restriction fragment length polymorphism (RFLP) patterns obtained from positive amplicons (see A, lanes 5 and 7: DMVce+ and CDVcr+) by enzymatic digestion with *MseI* is reported. RFLP analysis confirms specificity of positive reactions and differentiates amplified *N* gene target sequences in different genome morbillivirus types. Lanes 5–7: the same RFPL pattern of DMVce+ (referred to a DMV Mediterranean Sea genotype, accession no. HQ829973), all of which are different from the CDVcr+ RFLP pattern (referred to a CDV domestic carnivore genotype, accession no. KF914669), as described by Verna et al. (2017).

environmental pollutants, genetics, and immunologic dysfunction (Van Bressem et al. 2009).

In our study, the most frequent finding was nonsuppurative meningo-encephalitides in 30% of the animals harboring brain lesions. Furthermore, albeit at varying frequencies, all three major neurotropic pathogens investigated (DMV, *T. gondii*, *Brucella* spp.) were detected, in contrast to the study of Sierra et al. (2014), who reported only evidence of morbillivirus infection.

TABLE 1. Results of immunohistochemical (IHC) and molecular (PCR) testing for morbillivirus and *Toxoplasma gondii* in cetaceans stranded in Italy in 2002–14 and the associated neuropathologic lesions.[a]

| Case no. | Morbillivirus | | *T. gondii* | | Histology |
|---|---|---|---|---|---|
| | RT-PCR | IHC | PCR | IHC | |
| 1 | – | – | – | + | NSM |
| 2 | – | – | + | – | NSM |
| 3 | – | – | + | – | NSM |
| 4 | – | – | + | – | No lesions |
| 5 | + | + | + | + | NSM |
| 6 | – | – | + | – | Neoplasia |
| 7 | + | + | – | – | NSM |
| 8 | + | – | – | – | NSM |
| 9 | – | – | + | – | NSM |
| 10 | + | – | – | – | NSM |
| 11 | + | – | – | – | No lesions |
| 12 | + | + | + | + | Unsuitable |

[a] RT = reverse transcriptase; NSM = nonsuppurative meningo-encephalitides.

*Toxoplasma gondii* has often been considered a secondary pathogen for cetaceans (Dubey et al. 2008; Van Bressem et al. 2009; Mazzariol et al. 2012). These two cases of coinfection with morbillivirus could provide evidence supporting the hypothesis of immune system impairment linked to the simultaneous occurrence and development of morbilliviral infection (Kennedy 1998; Jauniaux et al. 2000; Van Bressem et al. 2009).

The finding of case with protozoan cysts in the absence of histologic lesions underlines the importance of examining the brain of stranded cetaceans, even if it is in poor condition, to detect potential inflammatory lesions or the occurrence of parasitic cysts. Cases (2–4, 6, 9) in which *T. gondii* was detected only by PCR, could be attributed the cysts not always being easy to highlight by histology and IHC, although additional histologic sections were cut. Similarly, the PCR

results did not confirm one of the IHC-positive cases (1), probably due to the low number of cysts present in the tissue.

Exposure to *Brucella* spp. has been described in numerous marine mammals stranded along the coasts of the UK (Jepson et al. 1997; Davison et al. 2017) and in other oceanic regions (Dawson 2005), but brucellosis has been confirmed as the cause of death in only a few cases. The occurrence of *B. ceti* associated with brain lesions was recently reported for the first time in the Mediterranean Sea in cetaceans found stranded on the Tyrrhenian Sea coast of Italy (Alba et al. 2013). Meningoencephalitis due to *B. ceti* has been frequently described in the literature (Foster et al. 2002; Gonzalez et al. 2002; Hernandez-Mora et al. 2008); however, no episodes of neuropathogenic *Brucella* spp. infection have been detected except for a case published by Garofolo et al. (2014), and our findings.

We found nonspecific lesions in seven brains, as no morbillivirus, *T. gondii*, or *Brucella* spp. infections were detected in these animals. Further studies would be useful to identify the origin of such lesions.

We diagnosed a glioblastoma in one dolphin stranded on the coast of Campania. Unfortunately, as not all brain areas were available, we were unable to determine the location and size of the tumor, which was histologically similar to a tumor described in an Atlantic spotted dolphin found stranded on the coast of the Canary Islands (Díaz-Delgado et al. 2015). Neoplasms in marine mammals are relatively uncommon, and their incidence is very low (Newman and Smith 2006). These data are probably underestimated because many deaths are not reported and many individuals die while still young; moreover, for logistic reasons, it is not always possible to sample or to collect the brains from stranded cetacean specimens. The etiology of most tumors in marine mammals is unknown.

We detected a suppurative lesion in only one brain sample. Bacteria are routinely detected in the respiratory and gastrointestinal systems of healthy captive marine mammals (Dunn et al. 2001). Many factors, such as morbillivirus infection, algal toxins, and contaminant accumulation, can play a role in the immune status of cetaceans and promote new or latent infections (Van Bressem et al. 2014). Among the many opportunistic or pathogenic bacteria isolated from marine mammals, some are also potential zoonotic agents (Higgins 2000). No bacteria were isolated from the brain sample that we examined, probably because the abscess was limited to the thalamus, which was not sampled.

Although no evidence of PrPSc deposition was detected by IHC in our samples, and in consideration of the reported detection of a prion-like disease in marine mammals (Di Guardo et al. 2012), we believed it was important to test all stranded animals for PrPSc (Di Guardo et al. 2012). Our results underscored the advantage of routine central nervous system sampling during necropsy, as it can yield scientific information relevant for cetacean health. Histopathologic and IHC examinations of suitable material are essential to establish correct morphologic and etiologic diagnoses.

Since marine mammals can be vectors of zoonotic agents, they represent an emerging public health issue. Furthermore, they are located at the top of the food web and are considered a sentinel species, acting as biologic indicators of the state of health of the sea and the surrounding environment. Our study highlights the need for additional research into the zoonotic potential of marine mammal pathogens and the etiology of neuropathologic conditions. An adequate surveillance system to monitor aquatic mammal pathologies is an important tool to protect both animal and human health.

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