Immunohistochemical investigations on Brucella ceti-*infected, neurobrucellosis-affected striped dolphins (*Stenella coeruleoalba*)*

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Brucella ceti, Immunohistochemistry, Neurobrucellosis, *Stenella coeruleoalba*.

Summary
Bacteria of the genus *Brucella* cause brucellosis, an infectious disease common to humans as well as to terrestrial and aquatic mammals. Since 1994 several cases of *Brucella* spp. infection have been reported in marine mammals worldwide. While sero-epidemiological data suggest that *Brucella* spp. infection is widespread globally, detecting *Brucella* spp.-associated antigens by immunohistochemistry (IHC) in tissues from infected animals is often troublesome. The present study was aimed at investigating, by means of IHC based upon the utilization of an anti-*Brucella* LPS monoclonal antibody (MAb), the central nervous system (CNS) immunoreactivity shown by *B. ceti*-infected, neurobrucellosis-affected striped dolphins. The aforementioned MAb, previously characterized by means of ELISA and Western Blotting techniques, was able to immunohistochemically detect smooth brucellae both within the CNS from *B. ceti*-infected striped dolphins and within a range of tissues from *Brucella* spp.-infected domestic ruminants. In conclusion, the results of the present study are of relevance both from the *B. ceti* infection's diagnostic and pathogenetic standpoints.

*Indagine immunoistochimica in esemplari di stenella striata (*Stenella coeruleoalba*)* Brucella ceti-*infetti con sintomi di neurobrucellosi*

Parole chiave
Brucella ceti, Immunoistochimica, Neurobrucellosi, *Stenella coeruleoalba*.

Riassunto
I batteri del genere *Brucella* causano la brucellosi, una malattia infettiva comune all'uomo e ai mammiferi terrestri e acquatici. Dal 1994 diversi casi d'infezione da *Brucella* sono stati segnalati nei mammiferi marini in tutto il mondo. I mammiferi marini infetti mostrano reperti lesivi analoghi a quelli osservati nei mammiferi terrestri con presenza di aborti, natimortalità, orchite e neurobrucellosi. Se da un lato i dati siero-epidemiologici suggeriscono che l'infezione da *Brucella* spp. è cosmopolita, la rilevazione mediante immunoistochimica di antigeni brucellari nei tessuti di animali infetti è spesso problematica. Obiettivo del presente studio è stato quello di valutare, mediante l'impiego di un anticorpo monoclonale nei confronti dell'antigene LPS di *Brucella* spp., l'immunoreattività del sistema nervoso centrale (SNC) in esemplari di stenella striata (*Stenella coeruleoalba*) *B. ceti*-infetti affetti da neurobrucellosi. L'anticorpo in questione si è dimostrato capace di riconoscere immunoistochimicamente le brucelle lisce sia nel SNC dei succitati animali *B. ceti*-infetti sia in più tessuti di ruminanti domestici *Brucella* spp.-infetti, essendo stato parimenti caratterizzato mediante ELISA e Western Blotting. In conclusione, i risultati di questo studio hanno rilevanza ai fini sia della diagnosi immunoistochimica sia della definizione patogenetica dell'infezione da *B. ceti*.

Brucella ceti was first isolated from an aborted bottlenose dolphin (*Tursiops truncatus*) fetus in 1994 (Ewalt *et al.* 1994) and, since then, several cases of infection have been reported among free-ranging cetaceans worldwide (Guzman-Verri *et al.* 2012). The first case of *B. ceti* infection in the Mediterranean was recorded only in 2009 (Isidoro-Ayza *et al.* 2014), with the first case of *B. ceti* infection having been recorded along the Italian coastline in 2012 (Alba *et al.* 2013). Besides the "classical" species, some recently discovered *Brucella* species have also demonstrated a zoonotic potential, as in the case of *B. ceti* (Whatmore *et al.* 2008, De Massis *et al.* 2019). *Brucella* spp. infection in marine mammals is characterized by a pathogenicity similar to that of terrestrial mammals. In addition, a documented involvement of the central nervous system (CNS) in the striped dolphin (*Stenella coeruleoalba*), similarly to what described in the human species, has been reported (Guzman-Verri *et al.* 2012), with neurobrucellosis having not been recorded in bovine, caprine, ovine, swine, or canine hosts. Nevertheless, this syndrome is a relatively common feature in non-treated human brucellosis-affected patients (Obiako *et al.* 2010). Therefore, cetacean neurobrucellosis may serve as an interesting comparative neuropathology and neuropathogenesis model to understand how the bacterium is capable to cross the blood-brain barrier, thereby giving rise to host's CNS invasion. While sero-epidemiological data suggest that *Brucella* infection is widespread globally (Nymo *et al.* 2011), detecting *Brucella* spp.-associated antigens by immunohistochemistry (IHC) in tissues from naturally or experimentally infected animals is often troublesome The present study was aimed at investigating, by means of an anti-*Brucella* LPS monoclonal antibody (MAb), the CNS immunoreactivity (IR) shown by *B. ceti*-infected, neurobrucellosis-affected striped dolphins, along with its comparative evaluation in a range of fetal tissues from *B. abortus*- and *B. melitensis*- infected ruminants.

A MAb raised against *Brucella* LPS was produced at Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise 'G. Caporale' (IZSAM) and characterized by Western blotting (WB) and indirect ELISA according to Di Febo and colleagues (Di Febo *et al.* 2012) and Portanti and colleagues (Portanti *et al.* 2006), being subsequently characterized against *B. abortus* RB51, *B. pinnipedialis* and *B. ceti*, which were not tested in the past experiments. Samples of lung, liver and placental tissues from 16 ovine fetuses originating from 15 ewes experimentally infected with *B. melitensis* biotype 3, along with samples of lung, liver and placental tissues from 6 additional aborted fetuses carried by sheep belonging to *Brucella*-free flocks, were preliminarly investigated, 20 years ago, against *Brucella* spp. The study was subsequently enhanced, in recent years, through the inclusion of 9 cases of *B. ceti* infection in striped dolphins, 8 of which were found stranded between 2012 and 2019 along the Italian coastline, while the remaining individual was found beached ashore in Canary Islands (Spain) in 2004.

The dolphin tissues were collected during post mortem examination, in tight agreement with the investigation protocols to be performed in the framework of the Italian National Stranding Network (INSN) for standard laboratory investigations on stranded cetacean specimens.

Positive and negative controls were included in each IHC run, with the positive ones being represented by the lung, liver and placental tissues from ovine and bovine fetuses either naturally or experimentally infected by *Brucella* spp. The brain from a Dolphin Morbillivirus-infected striped dolphin was additionally used as negative control tissue. Further negative controls were represented by tissue sections obtained from the 7 immunohistochemically positive, *B. ceti*-infected striped dolphins under study, from which the primary anti-*Brucella* Ab was omitted. More in detail, *Brucella* IHC was carried out using the MAb 4B5A against LPS *Brucella* diluted 1:10 to 1:100. Tissue sections were previously heat treated for antigen retrieval (at 121 °C for 8 minutes) in 0.01 M citrate buffer, pH 6.0. Immunoreactions were then visualized by means of a peroxidase technique (Envision Plus Kit, Dako at IZSAM and Vectastain elite ABC kit standard Vector at the Faculty of Veterinary Medicine, University of Teramo, Italy).

The *Brucella* spp. isolation and identification procedures were performed in accordance with the technique described in the OIE Manual of Diagnostic Tests and Vaccines (World Organisation for Animal Health 2017). With the only exception of the two individuals in wich *B. ceti* infection was diagnosed only by means of biomolecular and IHC techniques (ID 1.5, Table I). All the tissue samples

Table I. *Results of* Brucella ceti. IHC in the CNS from infected striped dolphins (*Stenella coeruleoalba*).

ID	IHC (IZSAM)	IHC (UniTe)	Bovine fetal lung tissue (positive control)
1_430 ES 2004	Positive	Positive	Positive
2_3479 IT 2012	Negative	Negative	Positive
3_4555 IT 2012	Positive	Positive	Positive
4_5566IT 2014	Negative	Negative	Positive
5_16769IT 2017	Positive	Positive	Positive
6_346IT 2017	Positive	Positive	Positive
7_202IT 2018	Positive	Positive	Positive
8_47465IT 2018	Positive	Positive	Positive
9_2785IT2019	Positive	Positive	Positive

Table II. *Indirect ELISA: cross-reactivities of MAb 4B5A anti-*Brucella *LPS.*

Bacterial strain	MAb 4B5A*
Brucella melitensis biovar 2	Positive
Brucella melitensis biovar 1 16M	Positive
Brucella melitensis biovar 1 Rev.1	Positive
Brucella abortus strain S19	Positive
Brucella abortus strain S99	Positive
Brucella abortus strain S99 (LPS)	Positive
Brucella abortus biovar 2	Positive
Brucella abortus biovar 3	Positive
Brucella abortus biovar 6	Positive
Brucella suis biovar 1	Positive
Brucella ovis	Negative
Brucella abortus RB51	Negative
Brucella ceti	Positive
Brucella pinnipedialis	Positive

were routinely processed for histopathology and *Brucella* immunohistochemistry (IHC), whose reliability and reproducibility were also evaluated by means of an 'inter-laboratory comparison', which involved two independent Pathologists (based at IZSAM and at Faculty of Veterinary Medicine, University of Teramo, Italy, respectively) (Table I).

The results of the characterization of 4B5A MAb are shown in Table II (i-ELISA) and Figure 1 (WB). Both i-ELISA and WB confirmed that the aforementioned MAb reacted with *Brucella* smooth strains, with the typical LPS-ladder pattern exhibited in WB analysis. Conversely, the same MAb did not react with rough *Brucella* strains (Table I). The results of IHC investigations are reported in Tables II and III. More in detail, *Brucella* spp.-associated antigens were detected in pulmonary necrotic cell debris as well as in the cytoplasm of both alveolar macrophages and neutrophils from the *B. abortus*-infected bovine fetuses (Figure 2A) as well as in liver cells from the *B. melitensis*-infected ovine fetuses under study (Figure 2B). Within the CNS from *B. ceti*-infected dolphins, macrophage-like cells were seen harbouring more or less consistent loads of microbial antigen (Figure 2, C and D). Neither background staining nor artifacts or positive IR were observed in negative control tissues.

The results obtained in the present study clearly showed a strong IR against *Brucella* LPS in tissues from all the *Brucella* spp.-infected, herein investigated bovine, ovine and striped dolphins (7 out 9 individuals) (Figure 2, C and D). In this respect, the negative IHC results observed in the CNS from 2 *B. ceti*-infected dolphins may be due either to the low sensitivity of *Brucella* spp. IHC when low bacterial concentrations are present in infected tissues, or to

Table III. Brucella melitensis *detection by immunohistochemistry in infected animals.*

Ovine tissues	Tested	IHC POS	Bovine fetal lung tissue (positive control)
Fetal lung	26	16	OK
Controls	6	0	OK
Fetal liver	26	16	OK
Controls	6	0	OK
Total	**64**	**32**	

the different neuro-topographical concentrations of *B. ceti* organisms, not coincident with that of the microscopic field under study. An additional factor to be considered refers to the experimental conditions used in a portion of this work, that are counterparted by the 'field conditions' under which post mortem examinations are routinely carried out on stranded cetacean specimens, including also the adverse effects exerted by post mortem autolysis.

Based upon the herein presented results, *Brucella* spp. IHC should be regarded as a laboratory procedure which is useful not only when analyzing

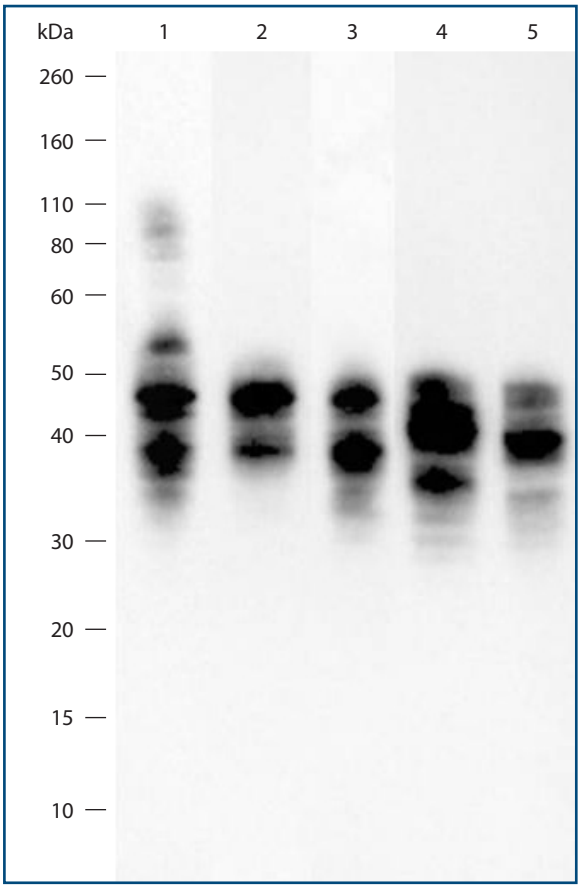

Figure 1. *Western blotting of Mab 4B5A vs* Brucella abortus *S99 (Lane 1),* Brucella melitensis *16M (Lane 2),* Brucella suis *biotype 1 (Lane 3),* Brucella ceti *(Lane 4),* Brucella pinnipedialis *(Lane 5).*

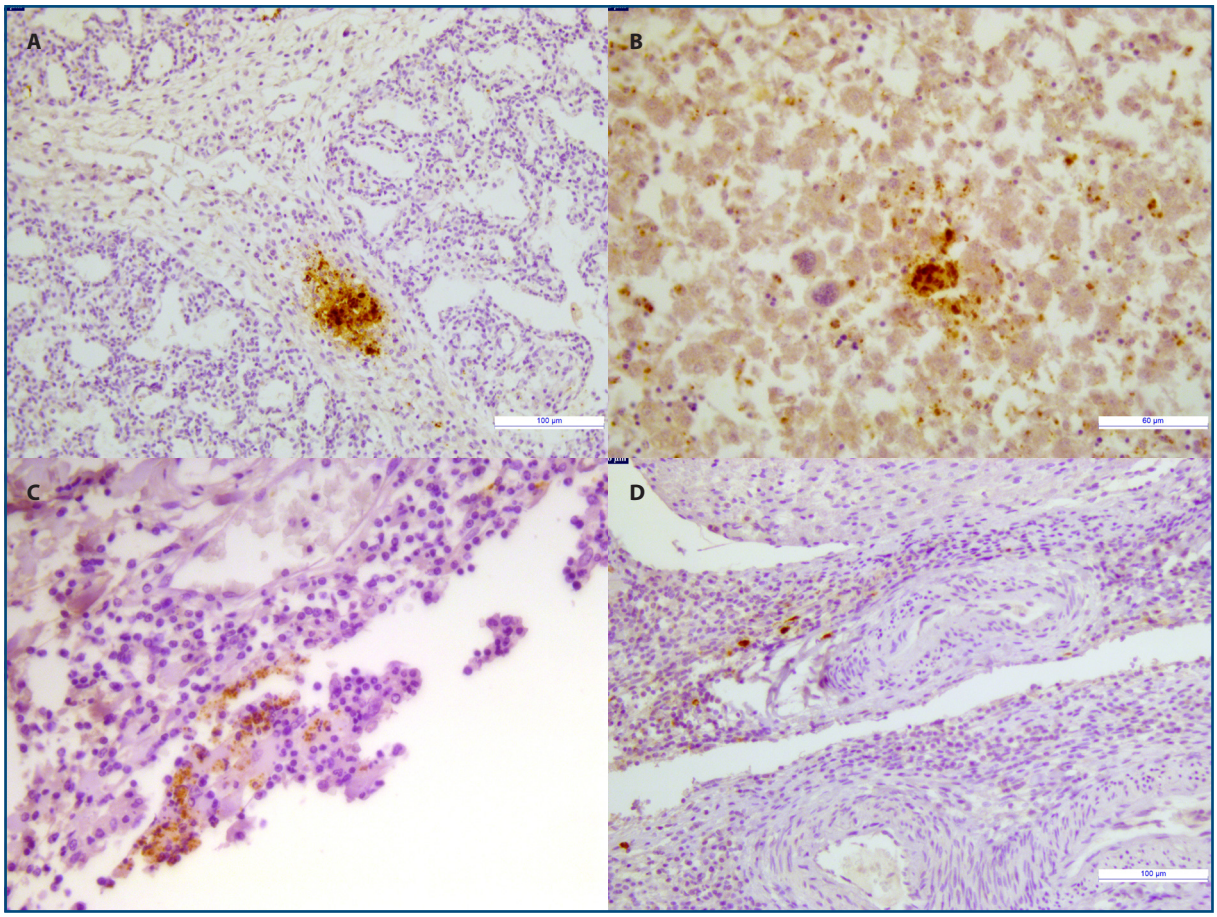

Figure 2. Brucella *spp.-associated antigen positive immunohistochemical labeling in bovine fetal lung (***A***), in ovine fetal liver (***B***) as well as in CNS (cervical spinal cord) tissues (***C***, ***D***) from neurobrucellosis-affected,* B. ceti*-infected striped dolphins. Brucella* spp. IHC with MAb 4B5A, Mayer's hematoxylin counterstain, different magnifications.

ovine and bovine infected tissues, but also in the case of *B. ceti*-infected, neurobrucellosis-affected striped dolphin CNS tissue specimens (in which macrophage-like cells were seen harbouring more or less consistent loads of bacterial antigen), providing a method capable of achieving a direct and reliable IHC diagnosis of *Brucella* infection. Furthermore, and not less important, the consistent background and the non-specific reactions observed when using an anti-*Brucella* -polyclonal Ab were not seen when MAb 4B5A was used.

The additional knowledge provided by this study on the detection of *Brucella* infection in cetaceans may be helpful not only from a diagnostic standpoint but also for increasing our awareness on the (neuro)pathogenesis of *Brucella* infection in aquatic mammals and, not less important, also from a public health viewpoint, considering the documented zoonotic potential of *Brucella* microorganisms. Moreover, MAb 4B5A anti *Brucella* LPS could represent a diagnostic and research laboratory reagent, whose use may be highly recommended also for the IHC diagnosis and pathogenetic characterization of *B. ceti* and *B. pinnipedialis* infections in cetaceans and in pinnipeds, respectively.

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