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

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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. Indeed, since human brucellosis ranks as one of the most common bacterial zoonotic infections on a global scale, it is necessary to increase our knowledge about it also in the marine environment. *Brucella ceti*, which is phenotypically similar to other smooth brucellae as *B. abortus* and *B. melitensis*, shares with the latter two the same surface antigens that are routinely used for the serological diagnosis of *Brucella* spp**.** infection. Marine mammal *Brucella* spp. infections are characterized by a pathogenicity similar to their terrestrial counterparts, with the occurrence of abortion, stillbirth and orchitis and an involvement of the host's central nervous system (CNS), similarly to what happens in mankind. 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 CNS immunoreactivity (IR) shown by *B. ceti*-infected, neurobrucellosis-affected striped dolphins.

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. Poiché la brucellosi umana è considerata una delle più comuni zoonosi su scala mondiale, è necessario migliorare le nostre conoscenze anche in ambiente marino. *Brucella ceti*, fenotipicamente simile ad altre brucelle lisce come *B. abortus* e *B. melitensis*, ha in comune con queste ultime i medesimi antigeni di superficie, che vengono peraltro utilizzati nella diagnosi sierologica dell'infezione. I mammiferi marini infetti ad opera di *Brucella* spp. mostrano reperti lesivi analoghi a quelli osservati nei mammiferi terrestri, uomo compreso, con presenza di aborti, natimortalità, orchite e neurobrucellosi. Se da un lato i dati siero-epidemiologici suggeriscono che l'infezione da *Brucella* spp. è praticamente cosmopolita, la rilevazione mediante tecniche d'indagine 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 in esemplari di stenella striata (*Stenella coeruleoalba*) *B. ceti*-infetti affetti da neurobrucellosi.

Parole chiave: Anticorpo monoclonale *Brucella ceti*, Immunoistochimica, Neurobrucellosi, *Stenella coeruleoalba.*

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 natural infection models in 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 *et al* (2012) and 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

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Moreover, you mention Fig. 2 before Fig. 1 in the text. Numbering of figures should be therefore revised.

105 diagnosed only by means of biomolecular and IHC techniques (ID 1,5 Table III). All the tissue
106 samples were routinely processed for histopathology and *Brucella* immunohistochemistry (IHC),
107 whose reliability and reproducibility were also evaluated by means of an "inter-laboratory
108 comparison", which involved two independent Pathologists (based at IZSAM and at Faculty of
109 Veterinary Medicine, University of Teramo, Italy respectively) (Table III).

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111 The results of the characterization of 4B5A MAb are shown in Table I (i-ELISA) and Figure 1 (WB).
112 Both i-ELISA and WB confirmed that the aforementioned MAb reacted with *Brucella* smooth
113 strains, with the typical LPS-ladder pattern exhibited in WB analysis. Conversely, the same MAb did
114 not react with rough *Brucella* strains (Table I). The results of IHC investigations are reported in
115 Tables II and III . More in detail, *Brucella* spp.-associated antigens were detected in pulmonary
116 necrotic cell debris as well as in the cytoplasm of both alveolar macrophages and neutrophils from
117 the *B. abortus*-infected bovine fetuses (Fig. 2) as well as in liver cells from the *B. melitensis*-infected
118 ovine fetuses under study (Fig. 3). Within the CNS from *B. ceti*-infected dolphins, macrophage-like
119 cells were seen harbouring more or less consistent loads of microbial antigen (Figs 4 and 5). Neither
120 background staining nor artifacts or positive IR were observed in negative control tissues.

121 The results obtained in the present study clearly showed a strong IR against *Brucella* LPS in tissues
122 from all the *Brucella* spp.-infected, herein investigated bovine, ovine and striped dolphins (7 out 9
123 individuals) (Fig. 3,4). In this respect, the negative IHC results observed in the CNS from 2 *B. ceti*-
124 infected dolphins may be due either to the low sensitivity of *Brucella* spp. IHC when low bacterial
125 concentrations are present in infected tissues, or to the different neuro-topographical concentrations
126 of *B ceti* organisms, not coincident with that of the microscopic field under study. An additional factor
127 to be considered refers to the experimental conditions used in a portion of this work, that are
128 counterparted by the "field conditions" under which *post mortem* examinations are routinely carried

Commentato [R2]: Table II: I would suggest to merge the columns "Tested" and "IHC POS" in order to present results as "16/26" for positives.
These 26 ovine tissues were previously tested as Brucella positive (so how do you explain negative results for 10?) or they were tested for the first time in this study?

Commentato [R3]: Table III: I would suggest to make explicit in the table the country and year of beaching (now It can only be inferred by the ID).

129 out on stranded cetacean specimens, including also the adverse effects exerted by *post mortem*
130 autolysis.

131 Based upon the herein presented results, *Brucella* spp. IHC should be regarded as a laboratory
132 procedure which is useful not only when analyzing ovine and bovine infected tissues, but also in
133 the case of *B. ceti*-infected, neurobrucellosis-affected striped dolphin CNS tissue specimens (in which
134 macrophage-like cells were seen harbouring more or less consistent loads of bacterial antigen),
135 providing a method capable of achieving a direct and reliable IHC diagnosis of Brucella infection.
136 Furthermore, and not less important, the consistent background and the non-specific reactions
137 observed when using an anti-*Brucella* -polyclonal Ab were not seen when MAb 4B5A was used.
138 The additional knowledge provided by this study on the detection of *Brucella* infection in cetaceans
139 may be helpful not only from a diagnostic standpoint but also for increasing our awareness on the
140 (neuro)pathogenesis of *Brucella* infection in aquatic mammals and, not less important, also from a
141 public health viewpoint, considering the documented zoonotic potential of *Brucella* microorganisms.
142 Moreover, MAb 4B5A anti *Brucella* LPS could represent a diagnostic and research laboratory
143 reagent, whose use may be highly recommended also for the IHC diagnosis and pathogenetic
144 characterization of *B. ceti* and *B. pinnipedialis* infections in cetaceans and in pinnipeds, respectively.

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150 Conflicts of Interest

151 The Authors declare that they have no competing interests in regard to this study.

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Figures' Legends

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

Brucella spp.-associated antigen positive immunohistochemical labeling in bovine fetal lung (Fig. 2), in ovine fetal liver (Fig. 3) as well as in CNS (cervical spinal cord) tissues (Figs 4 and 5) from neurobrucellosis-affected, *B. ceti*-infected striped dolphins. *Brucella* spp. IHC with MAb 4B5A, Mayer's hematoxylin counterstain, different magnifications.