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Atypical cerebral listeriosis associated with *Listeria innocua* in a beef bull

Paulo Ricardo Dell'Armelina Rocha[a]*, Alessandra Dalmasso[a], Carla Grattarola[b], Cristina Casalone[b], Fabio Del Piero[c], Maria Teresa Bottero [a], Maria Teresa Capucchio [a]

[a] *Department of Animal Pathology, University of Turin, Italy*
[b] *Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta, Turin, Italy*
[c] *School of Veterinary Medicine, Department of Pathobiology, New Bolton Center, University of Pennsylvania, PA, United States*

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A B S T R A C T

Natural infections of cattle associated with *Listeria innocua* have not been reported. This report describes the first case of cerebral listeriosis in a bull due to *Listeria innocua.* The animal presented neurological signs characterized by weakness, incoordination and recumbency. Histopathologic evaluation of brain tissue revealed multifocal microabscesses, perivascular lymphocytic cuffing, vasculitis, oedema and haemorrhages. All lesions extended from the medulla oblongata to the basal nuclei/parietal cortex area. Indirect immunohistochemistry labelled for *Listeria* sp. in the brain tissue, but not for *Listeria monocytogenes*, neurotropic Flaviviruses, BVDV, bovine Herpesvirus 1, *Chlamydophila* spp. and *Histophilus somni.* PCR was negative for ovine herpesvirus. *L. innocua* was isolated from brainstem and identified by biochemical tests (Camp and beta-hemolysis negative). Subsequently, the species was confirmed by a duplex PCR and minisequencing assays. *L. innocua* should be histologically considered as a differential diagnosis of thrombotic meningoencephalitis, malignant catarrhal fever and cerebral listeriosis due to *L. monocytogenes* in cattle.

1. Introduction

Listeria spp. are Gram-positive facultative intracellular bacteria ubiquitously distributed in the environment capable of growing at a wide range of pHs and temperatures (Vazquez-Boland et al., 2001). *Listeria* species are divided in two closely related lineages: *Listeria monocytogenes* and *Listeria innocua* form one group, while the second includes *L welshimeri, L. ivanovii*, and *L seeligeri* (Hain et al., 2006). Recently, two phylogenetically distant species *L mar-thii* (Graves et al., 2010) and *L rocourtiae* (Leclercq et al., 2010) were also described. Almost all cases of human listeriosis are due to *L monocytogenes;* very rare infections due to *Listeria ivanovii* and *Listeria seeligeri* have been described (McLauchlin and Martin, 2008). *L monocytogenes* is also the major pathogen for other animals, although approximately 10% of septicaemia in sheep has been reported as due to *L. ivanovii* (McLauchlin and Martin, 2008). Widespread in the environment and in food, *L innocua* is generally considered nonpathogenic (Vazquez-Boland et al., 2001). On the other hand, *L. innocua* has been associated with a human case of fatal sepsis and identified via blood culture and PCR assay (Perrin et al., 2003) and with a ewe that presented meningoencephalitis by brain bacterial culture and PCR assay (Walker et al., 1994). There are no detailed gross, histopathologic and molecular descriptions of fatal *L innocua* infection in cattle. The authors describe the neuropathology of one case of *Listeria innocua* meningoencephalitis in a bull in Northwestern Italy.

2. Materials and methods

An 18 months old Blonde Aquitaine bull presented neurological signs characterized by weakness, incoordination and recumbency. The animal died after 1 day and was referred to the Department of Animal Pathology of Turin University for a complete necropsy. During the post mortem, collected tissue samples were fixed in 10% neutral buffered formalin. Obex, pons, mesencephalon, cerebellum, hippocampus, thalamus, basal nuclei area, occipital, parietal and frontal cortex were transversally cut, routinely processed and embedded in paraffin. Five lm serial tissue sections were prepared for histopathologic and immunohistochemical examinations. Deparaffinized sections for histopathology were stained with hae-matoxylin and eosin (H&E).

Briefly, indirect immunohistochemistry was performed using primary antibodies against *Listeria* sp. (code 43251; Virostat, Portland, USA), *L monocytogenes* (code 223021; Becton Company, Sparks, USA), Arboflaviviruses (Bioreliance, Rockville, MD, USA), Bovine Viral Diarrhea Virus (BVDV) (provided by Dr. E. J. Dubovi, College of Veterinary Medicine, Cornell University, Ithaca), bovine

Herpesvirus 1 (VMRD, Pullman, USA), *Chlamydophila* spp. (Chem-icon, USA), and *Histophilus somni* (provided by Dr. J. Lopez, Univers-idad Nacional Autonoma de Mexico, Mexico City). For antigen retrieval, all but one brain tissues were incubated in Proteinase K; citrate buffer (pH 6.0) was used for slides incubated with primary *Listeria* sp. antibody. The secondary antibodies were: immun-operoxidase staining using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, USA) for *Listeria* sp. slides; DAKO LSAB2-HRP (DAKO, Carpinteria, USA) secondary kit detection system (biotin labelled goat anti-rabbit + goat anti-mouse) for *L. monocytogenes*, bovine herpesvirus 1 and *H. somni* slides; and DAKO EnVision™+/HRP kit (DAKO, Carpinteria, USA, code K5361) for Arboflaviviruses, BVDV and *Chlamydophila* spp. Subsequently, the $3,3^0$-diaminobenzidine chromogen (DAB) was incubated for 1 min at room temperature. Finally, the sections were counterstained using Mayer's haematoxylin. Malignant catarrhal fever (MCF) ovine Herpesvirus polymerase chain reaction (PCR) was performed from paraffin embedded tissues.

A fresh sample of the malacic area in the brainstem (Fig. 1a and b) was submitted for bacterial isolation in Blood and MacConkey Agar at 37 °C for 48 h. Subsequently, the same samples were placed in liquid media (Fraser broth and Demi-Fraser broth) and incubated at 37 and 4 °C for 48 h. Cultures were then streaked onto Listeria Oxford Agar (Microbiol, Italy) and incubated at 37 °C for 48 h. To identify the isolate, a miniaturized system was used (colorimet-ric Vitek 2 identification system, bioMerieux Inc, Bagno a Ripoli, Italy), as well as further CAMP and betahaemolysis tests.

For the confirmation of the species of the isolate, biomolecular assays (Dalmasso et al., 2010) were also performed from the broth cultures and from a frozen sample of the brainstem. DNA was extracted by boiling. A duplex PCR characterized by a specific fragment on phosphatidylinositol-specific phospholipase C (plcA) gene for *L. monocytogenes* (632 bp long) and by a common fragment of 16S rRNA gene for all *Listeria* spp. (437 bp long) was performed. When both bands were detected in the agarose gels, L. *monocytogenes* was immediately identified. When only the 437-bp band was present, it was possible to identify the *Listeria* genus but not to discriminate between the other *Listeria* species (L *innoc-ua, L. welshimeri, L. seeligeri, L. ivanovii,* and *L grayi).* In this case, the amplified product (437-bp band) was purified and submitted to a minisequencing reaction able to detect diagnostic sites that are able to identify five *Listeria* (already mentioned above; according to Dalmasso et al., 2010).

3. Results

At necropsy no significant macroscopic findings were noted in organs or tissues other than the brain. Transversal sections of the brain revealed dark brown areas interpreted as malacic foci involving the obex, pons, mesencephalon, thalamus and basal nuclei. Encephalomalacia was prominent in the obex and the right part of the pons. These lesions continued rostrally involving the right portion of the brain to the basal nuclei area as well as the adjacent parietal cerebral cortex (Fig. 1a-d).

Microscopically, lesions were moderate to severe, including multifocal microabscesses, vasculitis, perivascular lymphocytic cuffing, oedema and haemorrhages (Fig. 2a-d). Microabscesses were characterized by multifocal randomly distributed aggregates of neutrophils, small to coalescing foci of liquefactive necrosis and oedema. In the areas adjacent to the microabscesses there was multifocal gliosis, diffuse infiltration of neutrophils and microglial cells as well as neuronal necrosis. The microabscesses particularly involved the pons, cerebellum, thalamus and adjacent cerebral cortex. Necrotizing vasculitis with frequently associated perivascular haemorrhage was also observed within the brainstem, cerebellum, and white matter of the cerebral cortex. Perivascular cuffing was rarely observed in the obex, pons and midbrain and was composed of one to two layers of lymphocytes and occasional macrophages. Haemorrhages were observed in all areas of the brain, but were more severe in the cerebellum, thalamus and basal nuclei, where coalescing microabscesses were mostly observed. Multifocal moderate mononuclear leptomeningitis adjacent to microabscesses was also observed in the brainstem and cerebellum. Vagus, trigeminal and facial brainstem nuclei were affected by non-coalescing microabscesses and focal perivascular cuffing. Immunohistochem-istry for *Listeria* sp. allowed for the identification of the bacteria within the microabscesses, with the highest intensity noted in the cerebellum, thalamus (Fig. 2d) and mesencephalon. *Listeria* antigen was observed in the cytoplasm of mononuclear and

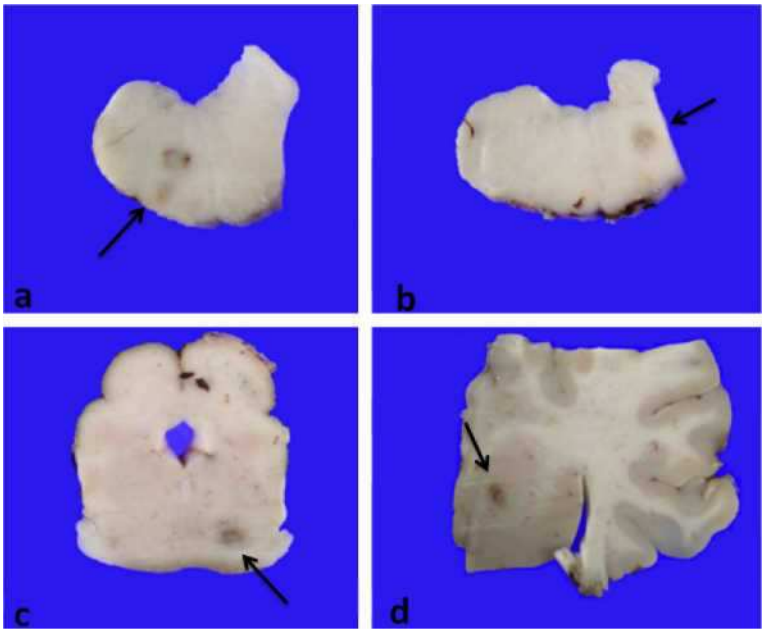

Fig. 1. (a) Brain, obex. (b) Brain, brainstem. (c) Brain, midbrain. (d) Brain, basal nuclei. Note the dark-brown malacic areas (arrows).

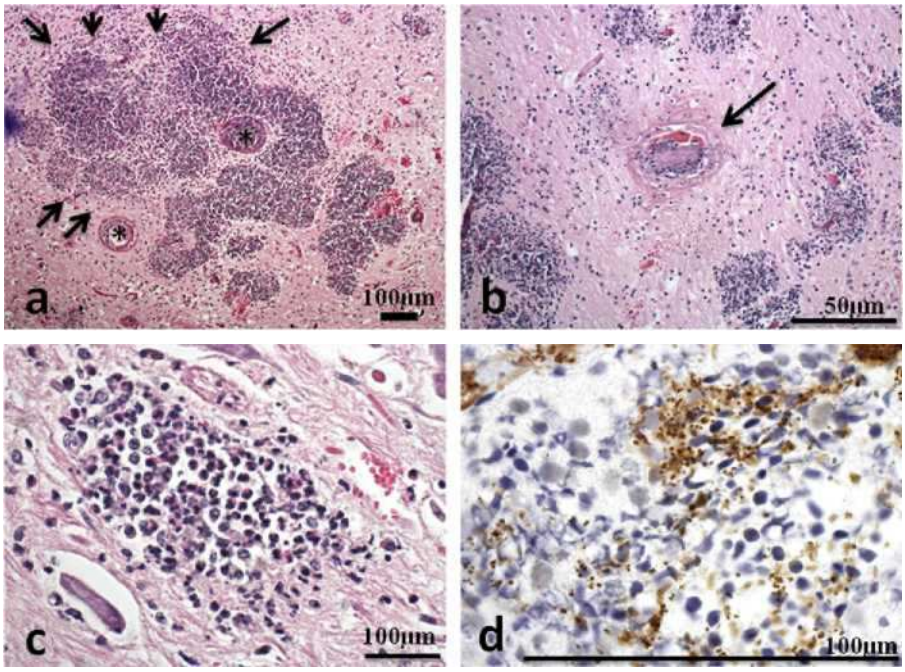

Fig. 2. (a) Brain, thalamus, coalescing microabscesses with malacic area (arrows) and vasculitis (asterisks) (HE). (b) Brain, thalamus, vasculitis (arrow) surrounded by multifocal to coalescing microabscesses (HE). (c) Brain, obex, microabscess affecting the vagus nuclei (HE). (d) Brain, cerebellum, brown areas correspond to immunopositivity for *Listeria* sp. antibody in a malacic area (DAB chromogen, counterstained with haematoxylin).

polymorphonuclear cells (resembling gitter cells and neutrophils, respectively) of microabscesses and rarely within the lumen of blood vessels affected by vasculitis. *L. monocytogenes*, neurotropic Flaviviruses, BVDV, bovine Herpesvirus 1, *Chlamydophila* spp. and *H. somni* were not present. MCF ovine herpesvirus was not detected by PCR.

L innocua was isolated only from the brainstem and the identity of the unusual isolate was confirmed by the Vitek 2 identification system and by Camp and betahaemolysis negative results. Duplex PCR (Fig. 3) of both the frozen brainstem sample and the bacteriology culture allowed the amplification of only a 16S rRNA gene 437-bp fragment in the isolate, indicating that this isolate was of *Listeria* genus, but not *L monocytogenes. L. monocytogenes, L. innocua* and other *Listeria* spp. present single nucleotide polymorphism in

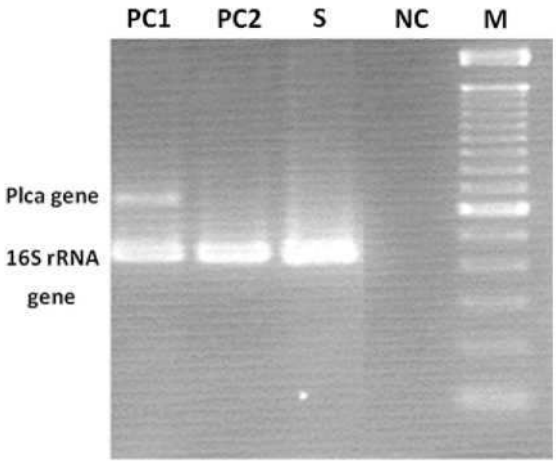

Fig. 3. Duplex PCR. M: marker, 100bp; PC1: positive control (L *monocytogenes);* PC2: positive control (L *innocua);* S: isolate of the present report, which amplified only a 467 bp fragment, as did PC2; NC: negative control (ultrapure water).

nucleotides 93, 178, 226, and 270 relative to *L innocua* 16S rRNA gene sequence (GeneBank Accession No. X56152). For bothL. *monocytogenes* and *L innocua*, nucleotides corresponding to these positions are A,G,G,T, respectively. These polymorphisms were detected with a minisequencing assay (Dalmasso et al., 2010). Together, minisequencing analysis result (data not shown) with the lack of amplification of the L. monocytogenes-specific fragment in the duplex PCR (Fig. 3) led us to conclude that the identified species was indeed *L innocua.*

4. Discussion

Bovine cerebral listeriosis is classically caused by *L monocytogenes*, while other species of *Listeria* have been traditionally classified as non-pathogenic (Low and Donachie, 1997). The microscopic lesions of the present case were similar to acute cerebral listeriosis due to *L monocytogenes* in ruminants (Oevermann et al., 2010). However, vasculitis in this instance was particularly severe and was often associated with large areas of necrosis and haemorrhage. Moreover, the microabscesses and areas of enceph-alomalacia were more evident and extensive than those described in the literature of typical cerebral listeriosis lesions in cattle (Ladds et al., 1974). Walker et al. (1994) described meningoencephalitis due to *L innocua* in a ewe in which histopathologic examination noted moderate mononuclear perivascular cuffing and neutrophilic margination. Vasculitis was also reported - as in the present case - but was restricted to the midbrain. Furthermore, lesions (Walker et al., 1994) were limited in distribution and severity in comparison with the present case and with cases of classical encephalitic listeriosis of sheep (Ladds et al., 1974), and microabscesses were not detected.

Other diseases, such as thrombotic meningoencephalitis (TBME) - caused by *H. somni* - or MCF can also cause vascular lesions similar to the present case in the brain of cattle (Summers et al., 1995; Maxie and Youssef, 2007). However, TBME generally causes severe vasculitis and thrombosis, with or without

infarction; there is a predilection for the thalamus and the junction of the white and gray matter of the cerebral cortex (Maxie and Youssef, 2007).

MCF infection is epidemiologically associated with cattle having contact with sheep or wild ruminants (Russell et al., 2009). There was no history of contact with these animals in this case. Additionally, MCF causes lymphocytic and histiocytic vasculitis with severe fibrinoid necrosis involving the tunica media and adventitia of the arteries and arterioles. Additionally, MCF in ruminants often causes erosive or ulcerative lesions in the gastrointestinal and respiratory tracts, as well as lymphoid hyperplasia (Summers et al., 1995), as opposed to the neutrophilic pathology associated with listeriosis.

Although Rocourt and Seeliger (1985) previously reported isolation of *L innocua* from the brain of cattle experimentally infected and concluded that it was non-pathogenic, the present paper constitutes the first description of natural infection, brain lesions, bacteriology, immunohistochemic positivity and molecular biology confirmation of L *innocua* infection in the brain of a bovine animal. Haemolysis is considered an important test to distinguish between *L. monocytogenes* and *L. innocua* (Gorski, 2008). However, misinterpretation from biochemical profiles of *Listeria* genus has been reported. Indeed, Johnson et al. (2004) reported that *L. innocua* may ocasionally present b-haemolytic activity, which is a virulence gene-related feature usually associated with *L monocytogenes*. Further molecular characterization of this isolate revealed the presence of internalin A virulence gene (Volokhov et al., 2007). Non-haemolytic strains of *L. monocytogenes* have also been described (Allerberger et al., 1997). Therefore, the species affirmation by Rocourt and Seeliger (1985), based only from biochemical characteristics, is uncertain.

Co-infection with *L monocytogenes* or other biological agents seems unlikely in this instance, since *L. innocua* was isolated from the brainstem. Finally, immunohistochemistry and PCR assays for other previously described pathogens were negative, ruling out the potential for co-infection. Although it was not possible to evaluate the immune status of the present bull, the authors suggest that this atypical infection may be caused by a combination of immunosuppression and a mutated bacterial strain. In fact, natural heterogeneity in avirulent *Listeria* species, reflected in its ability to acquire or retain virulence-associated genes, may permit some L. *innocua* to be causative agents of disease, especially in immunocompromised mammals (Volokhov et al., 2007).

To the best of the author's knowledge, this is the first description of fatal meningoencephalitis caused by *L innocua* in a bull. Therefore, the authors recommend to increase the use of microbiological and biomolecular techniques in all suspected cases of listeriosis additionally to the traditional histopathology and immunohistochemistry for a correct aetiological diagnosis. Furthermore, *L innocua* should be histologically considered as a differential diagnosis of TBME, MCF and cerebral listeriosis due to L. *monocytogenes* in cattle.

Conflict of interest statement

The authors declared that they had no conflicts of interest with respect to their authorship or the publication of this article.

References

Allerberger, F., Dierich, M., Petranyi, G., Lalic, M., Bubert, A., 1997. Nonhemolytic strains of *Listeria monocytogenes* detected in milk products using VIDAS immunoassay kit. Zentralblat Hygiene Umweltmed 200,189-195. Dalmasso, A., Rantsiou, K., Cocolin, L., Bottero, M.T., 2010. Development of a biomolecular assay for the identification of *Listeria* at species level. Foodborne Pathogens and Disease 7, 565-571. Gorski, L., 2008. Phenotypic Identification. In: Liu, D. (Ed.), Handbook of *Listeria monocytogenes.* CRC Press, Boca Raton, USA, pp. 162. Graves, L.M., Helsel, L.O., Steigerwalt, A.G., Morey, R.E., Daneshvar, M.I., Roof, S.E., Orsi, R.H., Fortes, E.D., Milillo, S.R., den Bakker, H.C., Wiedmann, M., Swaminathan, B., Sauders, B.D., 2010. *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. International Journal of Systematic and Evolutionary Microbiology 60,1280-1288. Hain, T., Steinweg, C., Chakraborty, T., 2006. Comparative and functional genomics of *Listeria* spp.. Journal of Biotechnology 126, 37-51. Johnson, J., Jinneman, K., Stelma, G., Smith, B.G., Lye, D., Messer, J., Ulaszek, J., Evsen, L., Gendel, S., Bennett, R.W., Swaminathan, B., Pruckler, J., Steigerwalt, A., Kathariou, S., Yildirim, S., Volokhov, D., Rasooly, A., Chizhikov, V., Wiedmann, M., Fortes, E., Duvall, R.E., Hitchins, A.D., 2004. Natural atypical *Listeria innocua* strains with *Listeria monocytogenes* pathogenicity island 1 genes. Applied and Environment Microbiology 70, 4256-4266. Ladds, P.W., Dennis, S.M., Njoku, C.O., 1974. Pathology of listeric infection in domestic animals. Veterinary bulletin 44, 67-74. Leclercq, A., Clermont, D., Bizet, C., Grimont, P.A., Le Flèche-Matéos, A., Roche, S.M., Buchrieser, C., Cadet-Daniel, V., Le Monnier, A., Lecuit, M., Allerberger, F., 2010. *Listeria rocourtiae* sp. nov. International Journal of Systematic and Evolutionary Microbiology 60, 2210-2214. Low, J.C., Donachie, W., 1997. A review of *Listeria monocytogenes* and listeriosis. The Veterinary Journal 153, 9-29. Maxie, M.G., Youssef, S., 2007. Nervous system. In: Maxie, M.G. (Ed.), Jubb, Kennedy and Palmer's Pathology of Domestic Animals, fifth ed. Elsevier, New York, USA, pp. 402-408. McLauchlin, J., Martin, W., 2008. Biology. In: Liu, D. (Ed.), Handbook of *Listeria monocytogenes.* CRC Press, Boca Raton, USA, pp. 4. Oevermann, A., Di Palma, S., Doherr, M.G., Abril, C., Zurbriggen, A., Vandevelde, M., 2010. Neuropathogenesis of naturally occurring Encephalitis caused by *Listeria monocytogenes* in ruminants. Brain Pathology 20, 378-390. Perrin, M., Bemer, M., Delamare, C., 2003. Fatal case of *Listeria innocua* bacteremia. Journal of Clinical Microbiology 41, 5308-5309. Rocourt, J., Seeliger, H.P., 1985. Distribution des espèces du genre Listeria. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. Series A 259, 317-330 (abstract in English). Russell, G.C., Stewart, J.P., Haig, D.M., 2009. Malignant catarrhal fever: a review. The Veterinary Journal 179, 324-335. Summers, B.A., Cummings, J.F., De Lahunta, A., 1995. Inflammatory diseases of the central nervous system. In: Summers, B.A., Cummings, J.F., de Lahunta, A. (Eds.), Veterinary Neuropathology. Mosby, St. Louis, USA, pp. 95-188. Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domfnguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* pathogenesis and molecular virulence determinants. Clinical Microbiology Reviews 14,584-640. Volokhov, D.V., Duperrier, S., Neverov, A.A., George, J., Buchrieser, C., Hitchins, A.D., 2007. The presence of the internalin gene in natural atypically hemolytic *Listeria innocua* strains suggests descent from *L monocytogenes.* Applied and Environment Microbiology 73,1928-1939. Walker, J.K., Morgan, J.H., McLauchlin, J., Grant, K.A., Shallcross, J.A., 1994. *Listeria innocua* isolated from a case of ovine meningoencephalitis. Veterinary Microbiology 42, 245-253.