FOODBORNE PATHOGENS AND DISEASE
Volume 6, Number 4, 2009
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DOI: 10.1089/fpd.2008.0226

Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from Muscle Tissue of Naturally Infected Cattle

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Abstract

Johne's disease or paratuberculosis is a chronic granulomatous inflammation of the small intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Recent studies suggest an association between MAP and Crohn's disease in humans. MAP can become widely distributed within the tissues of infected animals, and meat may be a possible route of exposure of MAP to humans. In this study, 47 dairy and beef cattle were examined for the occurrence of viable MAP in diaphragm muscle. At the slaughterhouse, gut tissues, diaphragm muscle, blood, and feces of the 47 animals were collected for bacteriological culture, as well as gut samples for histopathological analysis. MAP was detected by bacteriological culture and conventional and real-time IS900 polymerase chain reaction in the diaphragm muscle of six infected cattle at slaughter (13%). The six animals showing evidence of MAP in diaphragm muscle had diffuse lesions and severe granulomatous inflammation in ileocecal lymph nodes, jejunal lymph nodes, ileocecal valve, and ileum. All six had heavy bacterial load in mesenteric lymph nodes, ileocecal valve, ileum, and jejunum, and four showed clinical signs of paratuberculosis. Two animals did not show clinical signs but had viable MAP in intestinal tissues and in diaphragm muscle as well. MAP was found in blood of only one of the six animals showing evidence of MAP in diaphragm muscle and in feces of three of them. In general, there was a positive association between enteric lesion severity, clinical signs of paratuberculosis, heavy bacterial load in intestinal tissues, fecal shedding of MAP, and the presence of disseminated MAP infection in diaphragm muscle. The results of this study demonstrated that MAP can be detected and cultured from muscle of MAP-infected cattle destined for human consumption and suggest a possible risk of exposure of humans to MAP via contaminated meat.

Introduction

Paratuberculosis or Johne's disease is a chronic granulomatous inflammation of the small intestine of wild and domestic ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Paratuberculosis causes major economic losses to the cattle industry worldwide due to reduced milk production, lower weight gains, infertility, premature culling, and increased cow replacement costs (Ott *et al.*, 1999). Animals are most likely to be infected before 6 months of age, but clinical manifestations occur around 2 years of age (Chiodini *et al.*, 1984). Clinical signs of the disease include intermittent diarrhea, low milk production, and progressive weight loss that eventually results in death. Although the most important mode of transmission of paratuberculosis is by ingestion of contaminated manure, colostrum, water, or milk from infected cows, transmission can occur *in utero* and via infected semen as well (Ayele *et al.*, 2004). Recent studies have provided data suggesting an association between MAP and Crohn's disease in humans and the possibility that MAP could be transmitted to humans (Greenstein and Collins, 2004). Evidence supporting a link between MAP and inflammatory bowel disease in humans includes similarity between the clinical signs of Crohn's disease in humans and those found in animals with Johne's disease; detection of MAP by polymerase chain reaction (PCR) and by culture in feces, intestinal tissues, and peripheral blood of patients with Crohn's disease; association between MAP DNA in blood and cellular and humoral immune responses in inflammatory bowel disease patients; and anti-MAP antibiotic therapy resulting in reduction of bacteremia and remission, or substantial improvement in disease condition in many patients (Bull *et al.*,

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2003; Juste *et al.*, 2008a, 2008b). In spite of all of this evidence, it has still not been conclusively proven that MAP causes Crohn's disease. MAP may enter the food chain from a variety of sources. The organism, shed from cattle or from other animals, may contaminate pastures, and potable water, where it is resistant to standard purification with chlorine (Greenstein, 2003). Because MAP can survive pasteurization conditions, dairy products such as milk and cheese have been proposed as possible sources of exposure of humans to MAP (Chiodini and Hermon-Taylor, 1993). Since dairy and beef cattle infected with paratuberculosis are routinely sent to slaughter and their meat is used for human consumption, meat may be another possible route of exposure of humans to MAP (Grant, 2005). Although dissemination of MAP to liver, spleen, reproductive organs, kidney, and uterus via the blood and lymphatic vessels has been reported, no previous studies have detected MAP in muscle tissue of cows at slaughter (Antognoli *et al.*, 2008; Dennis *et al.*, 2008). The purpose of this study was to assess whether MAP may disseminate from its primary site of infection, the gastrointestinal tract, to the muscle tissue of naturally infected dairy and beef cattle.

Materials and Methods

Animals and collection of blood and feces

Forty-two dairy and five beef cows originating from eight dairy farms (Friesian and Jersey breeds) and two beef farms (Limousin breed) of the Basque Country, Spain, were slaughtered. The prevalence of MAP infection in these herds ranged between 3% and 10%. Cows were classified by the owners as having clinical signs consistent with paratuberculosis if they showed or had shown persistent diarrhea, weight loss, and/or low milk production, and/or had tested positive in paratuberculosis enzyme-linked immunosorbent assay (ELISA) or fecal PCR. On the day of slaughter each animal underwent standard antemortem clinical examination by slaughterhouse official veterinarians and blood and feces were collected for bacteriologic culture. Blood was collected from the jugular vein of each animal into 10-mL vacutainer EDTA tubes with 18-gauge needles.

Collection of tissue samples and histopathology

At the time of slaughter, approximately 200 g of diaphragm (thoracis) muscle was collected, the esophagus and rectum were cut, and all organs from the gastrointestinal system were removed from the carcass. We collected the diaphragm muscle due to its low commercial value and easy access (it lies between the abdomen and chest cavity). At the Biosecurity Lab at NEIKER-Tecnalia, an internal sample of diaphragm muscle was collected first from each animal to prevent potential cross-contamination with gastrointestinal contents. Subsequently, samples from ileocecal lymph node, jejunal lymph node, and a pool of samples from ileocecal valve (ICV) and terminal ileum were collected aseptically for histopathological examination. All instruments used for tissue collection were cleaned with 96% ethanol and flame-sterilized between tissues. Samples from areas showing gross lesions consistent with paratuberculosis, including thickened mucosa and enlarged lymphatic nodes were taken. Samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned with a microtome at 4 μm. All the sections were stained with hematoxylin and eosin and with Ziehl-Nielsen acid-fast stain. Paratuberculosis lesions were graded as focal, multifocal, intermediate diffuse, and multibacillary diffuse (González *et al.*, 2005).

Fecal culture

Collection and conventional culture of feces for isolation of MAP was performed as previously described (Juste *et al.*, 1991). Briefly, 2 g of feces were transferred into a stomacher bag, 38 mL of hexa-decyl pyridinium chloride (HPC) at a final concentration of 0.75% was added (Sigma, St. Louis, MO), and the material was homogenized in a stomacher blender. After 30 minutes of incubation at room temperature, 15 mL of homogenate was transferred to a new tube and incubated overnight for decontamination. Four drops of the suspension were inoculated by duplicate in Herrold's egg yolk medium (Becton Dickinson, Franklin Lakes, NJ) and in Löwenstein-Jensen medium (Difco, Detroit, MI), both supplemented with 2 mg/L of Mycobactin J (Allied Monitor, Inc., Fayette, MO). All four tubes were incubated at 37°C in a slanted position with loose caps to allow the surface of the medium to dry. Caps on the slants were tightened after 1 week, and cultures were observed every 4 weeks and considered negative if after 20 weeks no bacterial growth was observed. Samples were considered positive if one or more colonies were observed in one or more culture tubes. Isolated colonies were confirmed by PCR amplification of the MAP IS900 insertion sequence, as described below. The number of colony forming units (CFU) per culture tube was recorded for each positive sample. Animals with fecal culture colony counts were classified as low shedders (<10 CFU), medium shedders (10–50 CFU), and heavy shedders (>50 CFU). A CFU in each tube corresponded to approximately 40–80 CFU/g of feces (Sweeney *et al.*, 1992).

Tissue culture

A pool of samples from ICV and three sites of ileum (proximal, middle, and terminal ileum), a pool of samples from three sites of the jejunum (proximal, intermediate, and distal), and samples from ileocecal lymph node, jejunal lymph node, and diaphragm muscle were collected. For bacteriological culture, 2 g of each tissue sample was homogenized, blended, and decontaminated in 0.75% HPC and cultured on Herrold's egg yolk medium and on Löwenstein-Jensen medium slants in the same way as fecal culture (Juste *et al.*, 1991). Bacterial load was classified as low (<10 CFU), medium (10–50 CFU), and heavy (>50 CFU). Cattle were classified as negative if MAP was not isolated from feces, blood, or tissues. A cow was classified as infected with MAP if it had histological lesions consistent with MAP infection, or MAP detection by tissue culture was positive at slaughter.

MAP DNA isolation, PCR, and real-time PCR assays from muscle tissue

Isolation of MAP DNA from diaphragm muscle was performed using a MAP DNA extraction and purification commercial kit (QIAamp DNA Blood Mini Kit, Qiagen Gmbl, Hilden, Germany). Briefly, 2.5 g of tissue was put in sterile bags containing 10 mL of sterile water, homogenized in a stomacher blender for 2 minutes, and allowed to settle for 15 minutes. In a 2-mL microtube, 200 μL of the supernatant was

mixed with 40 μL of proteinase K (25 mg/mL) and 360 μL of SDS-containing lysis buffer (buffer ATL). After 1 hour at 55°C, the samples were lysed with 300 mg of glass beads, centrifuged, and filtered through Qiagen spin columns following the manufacturer's instructions. Purified DNA was tested by conventional PCR (Moss *et al.*, 1992) and by real-time PCR (Adiagene, Saint Brieuc, France) amplifications of the IS900 target sequence of MAP. For conventional PCR, two oligonucleotide primers (5′-GTT CGG GGC CGT CGC TTA GG-3′ and 5′-CCC ACG TGA CCT CGC CTC CA-3′) were selected to amplify a unique 390-bp fragment of the 5′ region of the MAP IS900 insertion sequence. For each PCR amplification, 5 μL of purified DNA was added to a 45-μL reaction consisting of 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each of dATP, dGTP, dCTP, and dTTP; 0.2 μM of each primer; and 2 U Taq DNA polymerase (Invitrogen, Carlsbad, CA). Conventional PCR was performed using the ABI Prism 2700 Detection system (Applied Biosystems, Foster City, CA) with the following conditions: one cycle of 96°C for 2 minutes; 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; and one cycle of 72°C 10 minutes. For each set of reactions, positive and negative controls were included. Ten microliters of the PCR amplifications were analyzed in a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. The real-time PCR system (Adiagene) uses a 6 carboxy-fluorescein (FAM)-labeled TaqMan probe and primers that specifically amplify a MAP IS900 sequence. An internal control was amplified in each reaction with a VIC-labeled TaqMan probe and specific primers. Real-time PCR was performed using the ABI Prism type 7000 (Applied Biosystems) with the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, and 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Only the samples with a typical amplification curve, a curve with a linear growing part followed by a plateau and with a Ct value below 40, were considered positive.

Results

Forty-two dairy and five beef cows originating from eight dairy farms and two beef farms of the Basque Country, Spain, were slaughtered due to clinical signs associated with paratuberculosis or for other reasons. As shown in Table 1, clinical signs of paratuberculosis including diarrhea, weight loss, and low milk production were observed by the owners in 12 of 47 (26%) of the slaughtered animals. Thirty-one of the 47 slaughtered cows (66%) were found to be infected with MAP by histopathology and/or bacteriological culture of gastrointestinal tissues. Of the 31 infected cows, 9 were Jersey (9 of 11 Jerseys), 17 Friesian (17 of 31 Friesians), and 5 Limousin (5 of 5 Limousin). Twenty-five of the 31 infected cows (81%) had infection limited to the gastrointestinal tract and associated lymph nodes. MAP was also detected by bacteriological culture, PCR, and real-time PCR from the diaphragm muscle of six infected cows (19%). The isolation of MAP from a non-gastrointestinal tissue, like the muscle tissue, suggested that these six animals had disseminated MAP infection. Culture results of diaphragm muscle, gut tissues, feces, and blood from the six animals with disseminated MAP infection and their ages and breeds are summarized in Table 2. The six animals showing evidence of MAP in diaphragm muscle had heavy bacterial load in ileocecal lymph node, jejunal lymph node, jejunum, ICV, and ileum, and three of them were shedding bacteria in feces. Four of the six animals with disseminated MAP infection had shown clinical signs associated with paratuberculosis according to the farmers (Table 2). The other two animals did not show clinical signs of paratuberculosis but had heavy bacterial load in gut tissues and low bacterial burden in diaphragm muscle. This result suggests that the presence of MAP in diaphragm muscle may also occur at an early stage of infection before the appearance of clear clinical signs of paratuberculosis.

In order to confirm the tissue culture results, PCR and real-time PCR of the six animals with viable MAP in diaphragm muscle were performed. Figure 1 shows the positive PCR and real-time PCR amplifications of four of the six DNA samples extracted from diaphragm muscles. Positive amplification of MAP DNA was obtained from diaphragm muscle of the six tested animals by the use of an IS900 real-time PCR (Adiavet Paratb real-time PCR) that had shown a 100% specificity and 85% sensitivity in detecting MAP and no false-positive signals with several non-MAP bacterial species tested.

Histopathological findings of the six animals showing evidence of MAP in diaphragm muscle by tissue culture and PCR analysis are summarized in Table 2. In the six animals, a clear association between extent and severity of enteric lesions and the occurrence of MAP in muscle was observed. All the animals with disseminated infection in muscle tissue had severe granulomatous enteritis, marked thickening and corrugation of the intestinal mucosa, notable enlargement of the associated mesenteric lymph nodes, and diffuse lesions positive by staining with Ziehl-Nielsen acid-fast stain in all the analyzed intestinal sections.

Table 1. Breeds, Clinical Signs, Histopathology, and Bacteriological Culture from Feces and Gastrointestinal and Muscle Tissues of 47 Slaughtered Cows

			Clinical signs (%)		*Histopathology (%)*				*Feces (%)*			*MLN (%)*		*ICV + ileum (%)*			*Jejunum (%)*		*Muscle (%)*	
Type	*Breed*	*No. of animals*	*PTBC*	*Other*	*Focal*	*Diffuse*	*Neg*	*nd*	*Pos*	*Neg*	*nd*	*Pos*	*Neg*	*Pos*	*Neg*	*nd*	*Pos*	*Neg*	*Pos*	*Neg*
Dairy	Jersey	11	45	54	27	45	27	0	56	45	—	82	18	73	27	—	73	27	9	91
	Friesian	31	22	77	16	29	45	10	22	74	3	32	68	36	61	3	32	68	10	90
Beef	Limousin	5	0	100	0	60	40	0	40	60	—	80	20	100	0	—	60	40	40	60
	Total	47	26	74	17	36	40	7	32	66	2	49	51	51	47	2	45	55	13	87

PTBC, paratuberculosis; Neg, negative; nd, undetermined; Pos, positive; MLN, ileocecal and jejunal lymph nodes; ICV, ileocecal valve.

Table 2. Breeds, Age, Histopathology, Clinical Signs, and Tissue Culture of Cows with Positive MAP PCR Result in Diaphragm Muscle

					Bacterial load[a]					
Animal code	*Breed*	*Age (years)*	*Histopathology*	*Clinical signs*	*Feces*	*Blood*	*MLN*	*ICV + Ileum*	*Jejunum*	*Muscle*
ES188589	Jersey	3	Diffuse paucibacillary	Positive	Medium	Negative	Heavy	Heavy	Heavy	Low
ES149682	Friesian	5	Diffuse multibacillary	Positive	nd	nd	Heavy	nd	Heavy	Heavy
ES134512	Limousin	7	Diffuse multibacillary	Negative	Negative	Negative	Heavy	Heavy	Heavy	Low
ES244438	Limousin	3	Diffuse intermediate	Negative	Medium	Negative	Heavy	Heavy	Heavy	Low
ES189242	Friesian	5	Diffuse intermediate	Positive	nd	Low	Heavy	Heavy	Heavy	Low
ES117687	Friesian	7	Diffuse intermediate	Positive	High	Negative	Heavy	Heavy	Heavy	Medium

[a]Bacterial load was classified as negative, low (<10 CFU), medium (10 to 50 CFU) and heavy (>50 CFU).
MAP, *Mycobacterium avium* subsp. *paratuberculosis*; PCR, polymerase chain reaction; MLN, ileocecal and jejunal lymph nodes; ICV, ileocecal valve; nd, undetermined.

Discussion

In our study, 31 of the 47 slaughtered cows (66%) were found by histopathology and/or bacteriological culture of gastrointestinal tissues to be infected with MAP. Of the 31 infected cows, 9 were Jersey (9 of 11 Jerseys), 17 Friesian (17 of 31 Friesian), and 5 Limousin (5 of 5 Limousin). These results agreed with previous studies showing some breeds of cattle (Jersey and Limousin) as being particularly susceptible to paratuberculosis (Clarke, 1997). We provide the first evidence that MAP can be detected and cultured from diaphragm muscle of MAP-infected cattle destined for human consumption. Even though the culture results do not raise any doubt and are the gold standard for paratuberculosis confirmation, we also carried out PCR detection in order to evaluate the potential of this much faster MAP detection method. PCR targeting the 5′ end of IS900 was considered specific for identification of MAP and has frequently been applied to confirm the presence of this organism in the diagnosis of Johne's disease (Green *et al.*, 1989). However, evidence showing that IS900-like elements existed in other environmental *Mycobacterium* species resulted in doubts concerning the use of MAP detection systems targeting this genetic element (Cousins *et al.*, 1999; Englund *et al.*, 2002). We used a commercial IS900 real-time PCR (Adiavet Paratb real-time PCR) for detecting MAP in muscle tissue of cattle. According to the manufacturer, this test has 100% specificity and 85% sensitivity in detecting MAP, and did not yield any false-positive signals on several non-MAP bacterial species tested.

Previously, only two skeletal muscle tissues, longissimus colli and extensor carpi radialis, cuts of which are often included in ground beef, were explored, and neither of them was culture positive for MAP in any of the tested animals (Antognoli *et al.*, 2008). Consumption of MAP-contaminated steak might represent a risk of exposure of MAP to humans, at least in raw or undercooked meat, even though no infection occurs. While meat heating should be effective in reducing numbers of contaminating MAP, the time and temperature to kill MAP when present in a meat matrix has not been determined. It should also be considered that low quality cow's meat is often used to manufacture meat products such as sausages and ground or minced meats, which could lead to the contamination of larger lots of the respective products.

Although previous studies reported disseminated MAP infection in several tissues (liver, kidney, spleen, gastrointestinal tract, and associated lymph nodes) of cattle with ad-

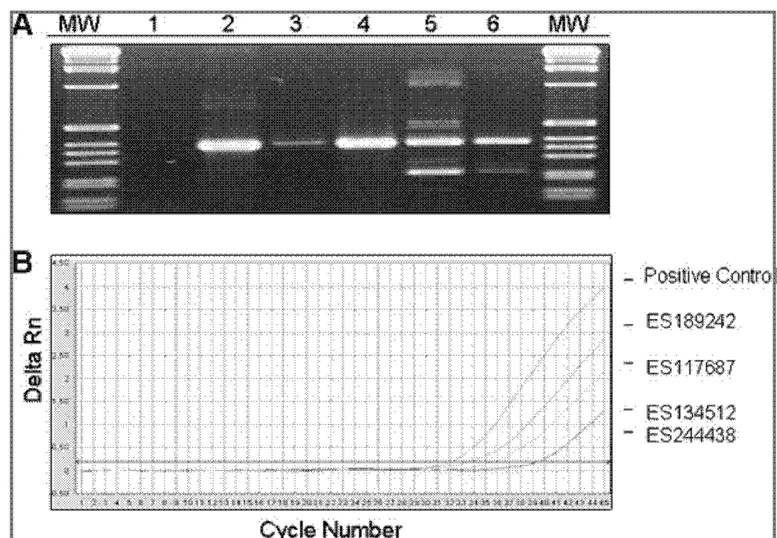

FIG. 1. IS900-specific polymerase chain reaction (PCR) and real-time PCR amplifications of DNA extracted from diaphragm muscle of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)-infected cattle. (**A**) PCR amplification. Lanes are as follows: Lane MW, molecular size marker; Lane 1, negative control; Lane 2, positive control; Lane 3, 4, 5, and 6, PCR amplifications of DNA extracted from diaphragm muscle of ES189242, ES117687, ES134512, and ES244438 cows, respectively. (**B**) Real-time PCR amplification plot from same samples as in panel A. Animals codes are shown in the left part of the image. Amplification of the internal control of each sample is not shown.

vanced clinical paratuberculosis, or in cattle shedding large numbers of MAP in feces (Whitlock and Buergelt, 1996; Pavlik *et al.*, 2000; Antognoli *et al.*, 2008), our study identified disseminated MAP infection in diaphragm muscle of both clinically and subclinically infected animals. Although the likelihood of having MAP in muscle is greater with more advanced disease, our results suggested that the presence of MAP in muscle tissue may also occur at an early stage of infection with no identifiable clinical signs of paratuberculosis.

The mechanism of MAP dissemination from its primary infection site, the gastrointestinal tract, has not been determined but is thought to occur through periods of sporadic bacteremia (Hines *et al.*, 1987). Following the uptake of MAP by M cells, subepithelial macrophages transport MAP trough the lymphatic system and MAP-infected macrophages may intermittently access systemic circulation (Koening *et al.*, 1993). Our study detected viable MAP in diaphragm muscle and blood from only one of the 47 slaughtered animals, probably due to sporadic access of infected macrophages to the blood and/or to the insufficient sensitivity of the technique. Bacteriological culture was positive in muscle and feces of three slaughtered animals. The fact that just some of the animals with disseminated infection were fecal shedders may be explained by a small quantity of organism shed, intermitting shedding, heterogeneous distribution of MAP in feces, or insufficient sensitivity of the fecal culture. A previous report indicated that a large percentage of animals affected with disseminated MAP infection escaped detection using serum ELISA or liver biopsy (Antognoli *et al.*, 2008). Thus, effective antemortem techniques should be developed to screen for disseminated MAP infection in cattle destined to human consumption.

Conclusion

Although our study is based on 47 cattle, which represent a comparatively small survey, it can be considered a representative size for the frequency found and provides new information about the extent of dissemination of MAP infection in Johne's disease–affected cattle. We found viable MAP in diaphragm muscle, a tissue remote from the gastrointestinal tract, in 6 of the 47 tested animals. Two of the positive animals did not show clinical signs associated with paratuberculosis and one was not confirmed as fecal shedder by fecal culture, suggesting that both symptomatic and asymptomatic animals could potentially represent a source of exposure of MAP to humans. Further research is needed to test more skeletal muscles commonly included in ground meat products for the presence or absence of MAP, to clarify the risk of exposure of humans to MAP via contaminated meat, and to develop more effective antemortem methods for identification of cattle with disseminated infection at slaughter, especially for subclinically infected animals. While further studies are underway, the food and cattle industry should adopt a precautionary approach and implement more effective control measures to reduce the chance of MAP contamination of foods and the entry of MAP-infected animals in the human food chain.

Acknowledgments

This study was funded by grants from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Spain) (RTA2005-00147-00-00) and from the Ministerio de Ciencia e Innovación (Spain) (AGL2006-14315-CO2). M. Alonso-Hearn's and J.M. Garrido's salaries were partially covered by the INIA program for incorporation of Ph.D. graduates into agricultural research centers. E. Molina has enjoyed a fellowship from the Foundation Candido Iturriaga y Maria Dañobeitia. P. Vázquez is a fellowship holder of the Ministerio de Ciencia e Innovación Formación de Personal Investigador (FPI) program. The authors thank the cattle herd owners for help in arranging slaughter of individual animals. We express our thanks to the veterinarians and staff at Donosti, Zestoa, and Bilbao slaughterhouses for help in collecting postmortem samples. We wish to thank the staff at the Laboratorio de Histopatología (NEIKER-Tecnalia) for assistance in performing formalin fixation of fresh tissues and in screening tissue sections. We thank Kyle P. Hearn for the editing of the manuscript.

Disclosure Statement

No competing financial interests exist.

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