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Detection of *Mycobacterium avium* subsp. *paratuberculosis* in kidney samples of red deer (*Cervus elaphus*) in Portugal: Evaluation of different methods

Ana C. MATOS[1,2)]*, Luis FIGUEIRA[2)], Maria H. MARTINS[2)], Manuela MATOS[4)], Sofia ÁLVARES[3)], Andreia MENDES[3)], Maria L. PINTO[1,3)] and Ana C. COELHO[1,3)]

[1)]CECAV, Centro de Ciência Animal e Veterinária, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal
[2)]Escola Superior Agrária, Instituto Politécnico de Castelo Branco, Castelo Branco, Portugal
[3)]Departamento de Ciências Veterinárias, Universidade de Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, 5000-801 Vila Real, Portugal
[4)]Departamento de Genética e Biotecnologia, Centro de Genómica e Biotecnologia, Instituto de Biotecnologia e Bioengenharia, Universidade de Trás-os-Montes e Alto Douro, Portugal

ABSTRACT. Paratuberculosis or Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is a chronic granulomatous enteritis affecting both domestic and wild ruminants. The present work is part of a wider set of studies designed to assess the prevalence of paratuberculosis in free ranging red deer (*Cervus elaphus*). With that purpose, 877 free-ranging red deer legally hunted in the Centre-eastern Portugal were submitted to necropsy and sampled for molecular methods, microbiology and histopathology. Thirty-seven (4.2%) kidneys revealed acid-fast bacilli when screened with the Ziehl-Neelsen technique. *Map* was detected by IS*900* polymerase chain reaction (PCR) in thirty (81.1%) of the Ziehl-Neelsen positive kidneys. Subsequent PCR and/or culture from the different organs of the 37 examined animals allowed us to detect 86.4% (32 animals) infected red deer. Our results suggest that renal involvement in *Map* infected deer may be underdiagnosed and thus the routine examination of this organ and its inclusion in PCR techniques designed for *Map* detection could substantially improve the diagnostic of paratuberculosis in red deer.

KEY WORDS: histopathology, kidney, *Mycobacterium avium* subsp. *paratuberculosis*, PCR, red deer

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Paratuberculosis (Johne's disease) is a chronic infectious disease affecting wild and domestic ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). The disease is prevalent worldwide and has a significant financial impact on those affected [16]. It has been also suggested that *Map* could be part of a causal structure or an opportunistic agent in human Crohn's disease, which is still being discussed [31].

In Europe, paratuberculosis infection has been reported in red deer (*Cervus elaphus*) [23, 25, 28], fallow deer (*Dama dama*) [20], roe deer (*Capreolus capreolus*) [28] and other wild species. Clinical signs of paratuberculosis in deer are similar to signs of the disease in sheep and cattle, with diarrhea, loss of weight and body condition [27].

The detection of mycobacteria by culture or molecular methods and the evaluation of histopathological lesions constitute the most effective methods for confirming a diagnosis of paratuberculosis [7, 21]. A diagnosis based on the detection of IS*900*-specific sequences of *Map* by polymerase chain reaction (PCR) from tissue, feces and blood is considered to be very quick and highly specific [6, 9, 11, 30] and according to Preziuso *et al*., 2012 [26], the most sensitive technique for detecting *Map,* especially in extra-intestinal samples.

The main objective of the present work was to assess the prevalence of paratuberculosis in free ranging red deer. For that purpose, the animals included in this study were submitted to necropsy and sampled for molecular methods, microbiology and histopathology. Typically, the small intestine and associated lymph nodes are the most important organs in the pathogenesis of paratuberculosis, but other organs may be also involved, a subject that has never been addressed in red-deer. Considering this aspect, it was deemed relevant to ascertain the usefulness of screening other organs for paratuberculosis-like lesions and *Map* detection. The data obtained with this study would improve the knowledge on the disease pathogenesis and diagnosis and could

*Correspondence to: Matos, A. C., Escola Superior Agrária, Instituto Politécnico de Castelo Branco, Quinta Srª de Mércules, Apartado 119, 6001-909 Castelo Branco, Portugal. e-mail: acmatos@ipcb.pt

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also help to prioritize the allocation of disease control resources in wild animals.

MATERIALS AND METHODS

Animals and samples

This study was based on 877 free-ranging red deer legally hunted in the Idanha-a-Nova (39° 55′ 11″ North, 7° 14′ 12″ West) and Penamacor (40° 10′ 8″ North, 7° 10′ 14″ West) cities (in Castelo Branco; Centre-eastern Portugal) during the period 2009–2011. Species were examined by a veterinarian. For animals that presented any visible gross lesion or animals that showed loss of weight or a rough coat, multiple tissues were collected and subjected to acid fast staining. The organs or tissues from animals that exhibited positive results were further investigated, and mycobacterial culture, polymerase chain reaction (PCR) and histopathological examination were performed. The samples consisted of retropharyngeal, mediastinal, bronchial, ileocecal and several mesenteric lymph nodes, palatine tonsil, lung, kidney, ileocecal valve and distal jejunum and ileum and urine collected at the post mortem examination. All tissues collected were removed using routine procedures developed to ensure that the likelihood of cross-contamination between samples and animals was minimized. Both kidneys from the 877 animals were tested by the Ziehl-Neelsen method.

All the samples were held frozen in individual sterile containers at −80°C until they were subjected to microbiological and PCR analyses. Date of collection, location and sex, as well as some particular features of the animals, were recorded. The age was estimated by the teeth eruption patterns: animals less than 12 months old were classified as juveniles, those between 12 and 24 months as yearlings and those more than two years old as adults [29].

Post mortem examination

The necropsy examination included detailed macroscopic inspection of lymph nodes and abdominal and thoracic viscera. This examination routinely included the parotid lateral and medial retropharyngeal and submandibular lymph nodes of the head. The tracheobronchial and mediastinal lymph nodes and lungs were examined within the thorax. Within the abdomen, the hepatic, mesenteric and ileocecal lymph nodes, ileocecal valve, liver, kidneys and spleen were examined. Any gross lesions in other locations were also recorded. Lymph nodes were dissected and sectioned serially. Imprint preparations of tissue samples were stained by the Ziehl-Neelsen (Z-N) method to detect acid-fast rods (AFRs). In each sample, at least 100 different fields were examined under an oil-immersion objective (100x). Tissue samples were fixed in a 10% neutral buffered formol-saline-solution by immersion. Fixed tissue samples were processed for histopathology using routine techniques for paraffin embedding. Tissues were sectioned at 4 μm and stained with hematoxylin and eosin (HE) and the Z-N technique. Microscopic lesions were evaluated regarding the presence and type of necrosis, the inflammatory infiltrate and the presence or absence of acid-fast organisms. Additional characterization of the lesions was performed when granulomatous lesions were present, including the staging of the granulomas according to previously described for cattle and fallow deer [12, 14, 32]. This classification system includes four stages: stage I (initial), stage II (solid), stage III (necrosis) and stage IV (necrosis and mineralization).

Culture

Culture methodology was performed as described by Juste *et al*.,1991 [15] and Aduriz *et al*., 1995 [1]. Samples from all animals were decontaminated with 0.75% (w/v) hexadecyl pyridinium chloride (HPC; Sigma-Aldrich, Milano, Italy) for 18 hr and cultured, in duplicate, using five specific media, supplemented with a mix of amphotericin B (50 mg/*l*), penicillin (100,000 U/*l*) and chloramphenicol (100 mg/*l*) (Sigma-Aldrich).

The five media used in this study were Löwenstein-Jensen medium (Liofilchem, Roseto degli Abruzzi, Italy), Löwenstein-Jensen medium with mycobactin J (*Synbiotics* Europe SAS, Lyon, France), Löwenstein-Jensen medium with sodium pyruvate without glycerol, Middlebrook 7H11 medium supplemented with OADC (oleic acid-albumin-dextrose-catalase) (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and Middlebrook 7H11 medium supplemented with OADC and sodium pyruvate without glycerol. All culture media were incubated at 37°C for 34–52 weeks and checked every week for mycobacterial growth or contamination with undesirable microorganisms. Colonies with compatible mycobacterial morphology were tested for acid fastness bacilli by the Ziehl-Neelsen stain of smears method. The mycobacterial isolates were tested for *Map* confirmation by the PCR methods described above.

DNA isolation

DNA isolation from kidney samples was carried out in duplicate by using a commercial DNA isolation kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) and stored at −20°C until used. DNA from bacteria isolated on kidney culture was extracted by taking a loop-full from a culture of Löwenstein-Jensen grown containing mycobactin, transferred to a microcentrifugal vial containing 100 μl of 10 mM Tris-HCl/ Triton X-100 1% / 1 mM EDTA (TTE) and incubated for 20 min at a temperature of 95°C. After centrifugation, the supernatant was stored at −20°C until used.

Polymerase chain reaction (PCR)

DNA from samples and from bacteria was tested in duplicate for *Map* using the primers RJ1 (GTT CGG GGC CGT CGCTTA GG) and PT91 (CCC ACG TGA CCT CGC CTC CA) flanking a region of 389 bp that were used for amplification of the IS*900* sequence of *Map* [13]. Each amplification reaction (final volume of 20 μl) was constituted by 3 μl DNA, 1 μl of each primer (10 μM), 10 μl Taq-PCR master mix (Qiagen) and 5 μl ultra-pure distilled water (Qiagen).

Table 1. Culture and PCR results in ZN+ kidney of *Cervus elaphus*. Additional granulomatous lesions, culture and PCR results from other tissues are also listed

Animals	*Map*		Kidney lesion	Granulomatous lesions in other tissues	*Map*	
n=37	PCR	Culture			PCR	Culture
1	+	–	-	-	+ MSLN	+ MSLN
2	+	–	Chronic interstitial nephritis	MSLN, MDLN, ICV, ICLN (stage III)	+ MSLN	-
3	+	–	Granuloma stage III, Chronic interstitial nephritis	MSLN, MDLN, ICV, ICLN, lungs (stage III and IV)	+ MSLN	-
4	+	+	Granuloma stage IV, Pyelonephritis	MSLN, MDLN, ICV, ICLN, RLN, lungs (stage III)	-	-
5	+	–	Granuloma stage IV	MSLN, MDLN, ICV, ICLN, RLN, lungs (stage III)	+ MSLN	-
6	+	–	Pyelonephritis	MSLN, MDLN, lungs (stage III)	+ MSLN	-
7	+	–	Granuloma stage III	MSLN, ICV, ICLN, lungs (stage III)	+ MSLN	-
8	+	–	Granuloma stage III, Chronic interstitial nephritis	MSLN, ICV, ICLN, lungs (stage III)	+ MSLN	-
9	+	–	Granuloma stage I	MSLN, ICV, ICLN (stage III)	+ MSLN	-
10	+	–	Granuloma stage I	MSLN (stage III)	+ MSLN	-
11	–	–	-	-	-	-
12	+	–	Chronic interstitial nephritis	MSLN, ICV, ICLN (stage III)	+ MSLN	-
13	+	–	-	MSLN (stage III)	+ MSLN	-
14	+	–	-	MDLN, lungs (stage III)	+ MSLN	-
15	+	–	-	MSLN (stage III)	+ MSLN	+ MSLN
16	+	–	Chronic interstitial nephritis	MSLN, ICV, ICLN, Lungs (stage III)	+ MDLN	+ MDLN; Lungs; ICV
17	–	–	-	MSLN (stage III)	-	-
18	+	–	Granuloma stage III, Chronic interstitial nephritis	MSLN, MDLN, Lungs (stage III)	+ MSLN	-
19	–	–	-	MSLN, ICV, ICLN (stage III)	-	-
20	+	–	-	MSLN, MDLN, ICV, ICLN (stage III)	-	-
21	+	–	-	-	+ MSLN	-
22	+	–	-	MDLN, Lungs (stage III)	-	-
23	+	–	-	-	+ MSLN	-
24	+	–	-	Lungs (stage III)	-	-
25	+	–	-	MSLN, RLN (stage III)	-	-
26	+	–	-	MSLN (stage III)	+ MSLN	-
27	+	–	Pyelonephritis, Chronic interstitial nephritis	MSLN, MDLN, ICV, ICLN (stage III)	+ MSLN	-
28	–	–	-	-	-	-
29	+	+	Granuloma stage III, Chronic interstitial nephritis	MSLN, ICV, ICLN (stage III)	+ MSLN	-
30	+	–	Chronic interstitial nephritis (perivascular nephritis)	MSLN, MDLN, Lungs, RLN, ICV, ICLN (stage III)	-	-
31	–	–	-	RLN (stage III)	+ MSLN	+ MSLN; MDLN; ICV
32	+	–	Pyelonephritis, Chronic interstitial nephritis	MDLN, ICV, ICLN (stage III)	+ MSLN	-
33	–	–	Pyelonephritis	MSLN, MDLN, Lungs (stage III)	-	-
34	–	–	-	MSLN, MDLN, Lungs (stage III)	+ MSLN	+ MSLN; MDLN; Lungs
35	+	+	Granuloma, stage III	MSLN, MDLN, Lungs (stage III)	+ MSLN	+ MSLN; Lungs
36	+	–	Granuloma, stage III	MSLN, MDLN, Lungs (stage III)	+ MSLN	+ MSLN; MDLN; Lungs
37	+	+	Granuloma, stage III	-	+ MSLN	+ MSLN

– negative; + positive; MSLN: mesenteric lymph nodes; MDLN: mediastinal lymph nodes; RLN: retropharyngeal lymph node; ICV: ileocecal valve; ICLN: ileocecal lymph nodes.

DNA amplifications were performed in a thermocycler with an initial step of 2 min at 96°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C, and a final 10 min extension at 72°C. Samples of 20 μl PCR products were analyzed on 1.0% agarose gels running at 90 V for 1 hr. The gels were stained using ethidium bromide. In addition to the samples, a positive (*Map* DNA) and a negative (water) preparation control as well as a blank control were included.

Data analysis

The association between the gross lesions in the organs and the infection status was analyzed. The animals were classified as infected if *Map* was isolated in the organs by culture or detected by PCR, and uninfected if no *Map* was isolated or detected. The severity of the gross lesions was quantified using the scoring system adapted from Zanella *et al.*, 2008 [33], described in Table 1.

RESULTS

A total of 877 free-ranging red deer were examined. Thirty-three (3.8%) animals out of 877 had gross lesions compatible with paratuberculosis in several organs (seven of these (0.79%) had gross lesions in kidneys). All kidneys from the 877 animals were screened by the Z-N method. Thirty-seven (4.2%) kidneys tested positive by Z-N of which eight (21.6%) were juveniles, eight (21.6%) were yearlings, and 21 (56.8%) were adults. The presence of *Map* in kidneys was confirmed by PCR in thirty (81.1%) out of the 37 kidney samples positive to the Z-N technique.

Gross lesions in mesenteric lymph nodes were detected in 31 (84%) animals out of the 37 studied animals. Seven (18.9%) had also gross lesions in the kidneys. The presence of *Map* in mesenteric lymph nodes was confirmed by PCR in 25 (67.6%) out of the 37 animals. Four of 25 animals showed no gross lesions.

Mycobacterium avium subsp. *paratuberculosis* was cultured from 27% studied animals in several organs (10/37), 10.8% of which (4/37) were kidney samples. All colonies obtained were positive by IS*900* PCR. Urine culture was negative in all the animals tested. Thirty two of the 37 animals could be considered as reliable infected cases of *Map* based on either of PCR or culture. Of the 32 infected animals, 71.9% had granulomatous lesions in the mesenteric lymph nodes, 46.9% in the lungs and mediastinal lymph nodes, 43.8% in the ileocecal valve and ileocecal lymph nodes, and 15.6% in the retropharyngeal nodes. The predominant lesion was the stage III granuloma, often multifocal, with a center of caseous necrosis, surrounded by inflammatory cells, amongst with epithelioid and multinucleated Langhans giant cells (Fig.1A and 1B). Liquefactive necrosis, calcification and the presence of neutrophils were the less frequent features. Due to advanced autolysis in most of the cases observed, the microscopic features of lesions in the ileocecal valve were more difficult to ascertain, but necrosis, flattening of the villi and the inflammatory infiltrate with epithelioid macrophages were frequently observed.

Lesions found in the Z-N positive kidney smears from *C. elaphus* are displayed in Table 1 and varied from granulomas of solitary or multifocal nature to chronic interstitial nephritis and chronic pyelonephritis (Fig. 1A to 1F). Table 1 lists the lesions from cases that yielded positive results for *Map* in PCR and culture from kidney tissues as well as the lesions, culture and PCR results from other organs of the same animals. In the kidney, lesions consisted mainly of granulomas, which varied in size from microscopic to up to 1 cm in diameter in the cases with a solitary lesion. In most of the cases, the lesions were stage III granulomas, with a core composed of necrotic debris of caseous nature (10.8%), surrounded by inflammatory cells, namely macrophages, and occasional epithelioid macrophages, lymphocytes (16.2%), plasma cells, but also neutrophils (13.5%). Langhans giant cells (13.5%) were present in the granulomatous lesions, but calcification was absent, except for one case. Only a small percentage (5.4%) presented liquefactive necrosis at the center of the granulomatous lesion. Granulomas were associated with chronic interstitial nephritis in some of the cases observed. *Map* was detected in one case that presented chronic interstitial inflammatory infiltrate with marked perivascular location (Fig. 1E). Chronic pyelonephritis was also observed in one animal that tested positive for *Map* (Fig. 1F). In other organs, multifocal type III granulomas were also the prominent lesion, but generally with a larger necrotic center than the granulomas observed in the kidneys. No mycobacteria were visualized in the organ samples submitted to histopathological examination.

From the 30 animals with kidney positive results for *Map* on PCR, generalized disease, corresponding to a score of 4 according to the classification by Zanella *et al*., 2008 [33], was observed in 18 (60.0%) infected animals. With a score of 3, two (6.7%) animals had lesions in the lungs with or without the involvement of abdominal organs. From the 4 animals with positive results for *Map* on kidney culture, a score 4 was observed in 3 animals (75%) (Table 2).

DISCUSSION

Mycobacterium avium subsp. *paratuberculosis* is one of the most common causal agents of mycobacterial infection in deer [2]. *Map* has been previously isolated from hepatic lymph nodes and spleen in deer [17], but as far as we know, this is the first time it has been detected in deer kidneys, and it is the first report of the disease in the free-ranging population in Portugal. In fact, *Map* in kidneys was previously reported in cows with advanced paratuberculosis [24], however, it was not referred to in wild deer. In this species, our data suggest that the involvement of kidney also occurs in advanced cases of the disease.

According to our observations, the classification parameters for paratuberculosis lesions suggested for other species appear to be valid and applicable to red deer [3]. The results of previous studies suggest that *Map* infection in red deer has an important role in their mortality and in calf mortality [27]. Paratuberculosis was confirmed in 32 of the 37 deer using histopathology, molecular and microbiological methods. Necropsy revealed gross lesions in 33 animals. The gross and microscopic lesions observed were consistent with those previously documented [5, 7, 21]. Our results agree with previous studies that suggest that smears stained with Ziehl-Neelsen are good indicators of infection and could be used to easily and rapidly screen tissue for the presence of compatible organisms that could be confirmed later by other methods with the added advantage of being inexpensive and fast [8].

Map was previously detected in kidneys in cows with advanced Johne's disease [24]. The jejunum, ileum and associated lymph nodes would seem to be the site of choice for seeking evidence of paratuberculosis in deer [3]. However, kidneys appear as an organ of choice too. Based on the lack of knowledge/information regarding which *Mycobacterium* could be found in samples, we used 5 different culture media in order to increase sensitivity by meeting the growth requirements of diverse *Mycobacterium*.

Although no significant associations were found between scores and PCR in kidneys, our results show a tendency of kidney involvement, with kidney lesions, in generalized and advanced forms of paratuberculosis, in which multifocal type III granulomas in lymph nodes and other organs are observed, and PCR positive results in mesenteric lymph nodes are registered. The

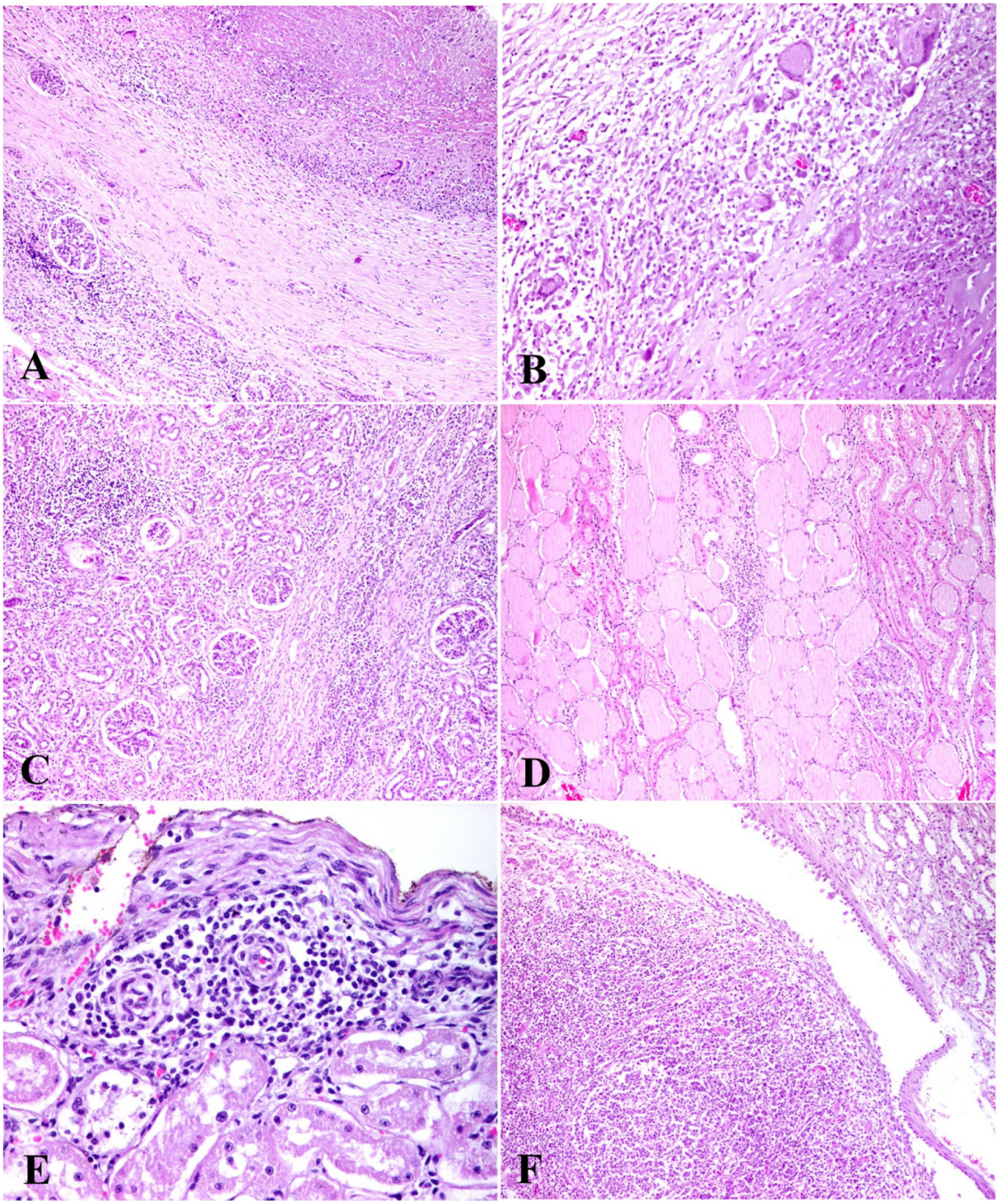

Fig. 1. Histologic features of the kidneys lesions observed in *Map* infected *C. elaphus*. Granulomatous lesions with a center composed of caseous debris surrounded by macrophages, epithelioid cells, Langhans multinucleated giant cells, lymphocytes and plasma cells (A and B). Chronic interstitial nephritis with lymphocytes, plasma cells and macrophages in the renal cortex (C); some animals, with chronic interstitial nephritis also displayed hyaline cilinders in the proximal and distal convoluted tubules (D); in other animals, the interstitial nephritis assumed a perivascular location (E). Chronic pyelonephritis with lymphocytes, plasma cells and macrophages infiltrating the renal pelvis. H-E staining; Original magnifications: A, C, D and F, × 10; B, × 20; E, × 40.

Table 2. Distribution of scores for diversity of paratuberculosis-like lesions and PCR positive in kidneys in red deer in Portugal

Score	Characteristics of lesions [a]	Kidney PCR positive (n; %) [b]	Kidney culture positive (n, %)
1	No visible lesions	1/2 (50.0)	0/2
2	Lesions in at least one type of lymph node (retropharyngeal, pulmonary such as tracheal, bronchial and mediastinal lymph nodes or mesenteric) and no lesions in internal organs	9/11 (81.8)	1/11 (9.1)
3	Lesions in internal organs (lungs, liver, kidney)	2/2 (100)	0/2
4	Lesions in at least one type of lymph node (retropharyngeal, pulmonary or mesenteric) and lesions in internal organs	18/22 (81.8)	3/22 (13.6)
Total		30/37 (81.1)	4/37 (10.8)

a) Adapted from Zanella *et al.*, 2008 [33]. b) No significant associations were found between scores and PCR in kidneys.

granulomatous lesion, in particular, stage III granulomas are the most common lesion, but an interesting feature is the presence of chronic interstitial nephritis alone or associated with the granulomatous lesion in *Map* positive animals. PCR in kidneys was positive in one deer, while negative in both histopathology and culture. This particular finding points to the fact that in some cases, kidneys may have sufficient bacterial DNA to allow identification by PCR, even when tissue culture and histopathology are negative. On the other hand, a plausible explanation for this occurrence is that the same piece of tissue cannot be used simultaneously for histopathology, culture and PCR, and that the portion saved for microbiological and histopathological purposes might not contain mycobacteria or lesions.

Secondly, PCR does not require the presence of viable organisms, whereas it is necessary for bacterial culture [22]. PCR has several advantages over current microbiological methods, such as speed, simplicity and no necessity for viable organisms [10]. However, it can fail to detect the agent in a significant proportion of animals with focal lesions [3]. This may be due to the inability to detect small numbers of mycobacteria or to the presence of unusual forms of mycobacteria [3]. Several authors have reported that in contrast to clinical symptoms of the disease in cattle, paratuberculosis in deer may often be clinically diagnosed in younger rather than older animals, in which severe emaciation and occasionally mortality are observed. Older animals appear to become more resistant to the infection and are less susceptible to the disease [17–19]. However, in our study, we found more infected adults. These results are important, because age classes may influence the epidemiology of paratuberculosis in deer [19]. A recent study on the Iberian Peninsula suggests that it is unlikely that wild red deer make a significant contribution to the continuation of *Map* infection in the region [5]. However, in Spain, a serological study in red deer population revealed that 30.2% of the animals sampled were positive [27]. Red deer are an economically important game species in Portugal [4] and share pastures and waterholes with sheep, goats, cows and a variety of wild animals, such as foxes and wild boar, and thus, the study of paratuberculosis' prevalence should be regarded as a matter of great epidemiological significance.

Our study suggests that renal involvement by paratuberculosis infection may be underdiagnosed in red-deer (*C. elaphus*). The routine examination of these organs as well as their inclusion in PCR techniques designed for *Map* detection could improve the diagnosis of paratuberculosis in this species. In fact, and being aware that cost-benefit and time are important factors in a diagnostic laboratory, the combined results of histopathology and PCR in kidneys obtained in the present study suggest that they represent effective tools in the diagnosis of paratuberculosis, as they are fast and relatively inexpensive to perform.

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