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Renal histopathological changes in dogs naturally infected with *Ehrlichia canis*

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Abstract

Renal involvement in Canine Monocytic Ehrlichiosis (CME) has been demonstrated in chronic cases without histopathological classification of patterns of glomerulopathy. Thus, in this study we proposed to evaluate the histopathological pattern, focusing on the types of glomerulonephritis in kidneys of dogs naturally infected with *Ehrlichia canis*.Twelve dogs naturally infected with *E. canis* and six healthy dogs were used. After clinical evaluation and diagnostic confirmation, the animals were euthanized and kidney fragments were obtained for histopathological examination, including hematoxylin and eosin (HE), Masson's trichrome, periodic acid-Schiff (PAS), periodic acid-methenamine silver (PAMS) and Congo red stain. Histopathological analysis of dogs with CME demonstrated that major lesions were present in the glomerulus and tubulointerstitial region in 100% of cases. The type of glomerular injury was membranoproliferative in 83.33%, and proliferative in 16.67%. Interstitial nephritis was present in the cortical region of all infected dogs. Minimal to marked lymphohistioplasmacytic inflammatory infiltrate was present in the interstitial, perivascular and periglomerular areas. Differential count of inflammatory cells indicated that lymphocytes predominated in comparison to plasma cells and histiocytes. Interstitial fibrosis and hyaline casts of lower intensity were observed. There was a negative correlation between serum albumin levels and severity of glomerulopathy. In conclusion, membranoproliferative glomerulopathy and interstitial nephritis are the major renal lesions in CME. It has been suggested that the presence of inflammatory infiltrates rich in lymphocytes indicates participation of these infiltrates in the immunopathogenesis of renal lesion in dogs with CME. Furthermore, hypoalbuminemia also seems be a marker of renal damage in dogs infected with *E. canis*.

Key words: dog, *Ehrlichia canis*, kidney, histopathology.

Introduction

Canine Monocytic Ehrlichiosis (CME) is considered one of the main infectious diseases in dogs (34). It is a cosmopolitan disease with a higher prevalence in areas of tropical and subtropical climate (47). The causative agent of CME is *Ehrlichia canis*, an obligate intracellular bacillus that infects mononuclear cells and disseminates throughout various tissues of the host (11). It is a tick-borne disease and the vector responsible for transmission is the *Rhipicephalus sanguineus* (11). Although primarily pathogenic to dogs, the pathogen also has a zoonotic potential (32).

The disease is characterized by three phases: acute, subclinical and chronic. Clinical manifestations are nonspecific: apathy, fever, vomiting, skin lesions, ocular and nasal secretion, splenomegaly, pale mucosa, lymphadenopathy, and hemorrhage. In severe cases, damage to the bone marrow may occur with pancytopenia, leading to death of the animal (5, 14, 28, 38). Laboratory abnormalities including anemia, thrombocytopenia, hypoalbuminemia, and hyperglobulinemia are commonly

associated with *E. canis* infection. Increased serum blood levels of urea, creatinine and phosphorous have also been cited (44).

Despite many existing research studies that contribute to the elucidation of mechanisms involved in the pathogenesis of CME, there are still few studies focused on renal disease in dogs with *E. canis* infection. It is known that in the acute phase, infected circulating mononuclear cells invade mainly the lungs, kidneys, and meninges, adhering to the vascular endothelium leading to vasculitis and subendothelial tissue infection (39).

Renal failure is not commonly associated with ehrlichiosis. However, studies have demonstrated that deposition of immune complexes in the kidneys may trigger glomerulonephritis and predispose the animal to proteinuria (15, 17). Renal failure was characterized in the experimental dog infection with *E. canis*. These animals developed transitory proteinuria, with loss of albumin, during two or three weeks following infection (10). In some cases, increased serum concentrations of urea and creatinine were also observed, suggestive of prerenal azotemia (3) and/or glomerulopathy in chronic cases (40).

Major histopathological findings are characterized by multifocal chronic glomerulonephritis (14) with lymphoplasmacytic infiltrate (9, 10). Furthermore, perivascular plasma cell infiltrate has also been reported in the corticomedullary junction, perivenular/periglomerular edema and focal necrosis followed by cellular infiltration (20, 33). It is believed that infection due to *E. canis* may lead to amyloidosis-associated kidney disease (25).

Renal damage is evident in chronic cases of dogs with CME. However, the morphological characterization of renal lesions, particularly glomerulopathy, has not been fully explored. Thus, we were the first to propose a detailed study of the histopathological pattern emphasizing the type of glomerular lesion in dogs naturally infected with *E. canis* from the endemic area of Teresina-PI. We showed that renal disease in CME is characterized by both glomerular and tubulointerstitial lesions. The predominant pattern of lesion was membranoproliferative glomerulonephritis.

Material and methods

Animal screening

Eighteen male and female mixed-breed adult dogs, of unknown age were selected from the Zoonosis Control Center in Teresina (State of Piaui, Brazil) for this study. Twelve infected dogs with *E. canis* and six healthy dogs were evaluated. Inclusion criteria for positive dogs were serological and *nested*-PCR (nPCR) molecular test positive for ehrlichiosis, and negative for leptospirosis (SAM) and visceral leishmaniasis (direct test, DPP, ELISA and immunohistochemistry). Noninfected control animals were stray dogs, which were captured for sacrifice due to rabies control. These animals were kept under the usual housing conditions (i.e., housing, food, temperature regulation, and ventilation) at the Zoonosis Control Center in the city of Teresina, until euthanasia. Control dogs were asymptomatic and negative in all diagnostic tests performed.

After physical examination, 5 mL of peripheral canine blood obtained by venous cannulation were placed in EDTA anticoagulant tubes (BD Vacutainer) for hematological analysis and molecular diagnosis of *E. canis*. Another 10 mL of peripheral blood were drawn and placed in tubes without anticoagulant (BD Vacutainer) for serologic and biochemical analysis. The animals were sacrificed for the collection of kidney fragments destined for histopathological test, immunohistochemistry and DNA detection of *E. canis*.

Ethical considerations

Experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal do Piauí (protocol numbers 070/12, 026/14, and 076/14), in compliance with guidelines formulated for procedures related to animal euthanasia, established by the Federal Council of Veterinary Medicine, Resolution number 714, in June 20, 2002.

Diagnosis of CME

Serology - ELISA (Immunocomb-ELISA, Biogal, Israel) was used to detect *E. canis* IgG antibodies. The technique was performed according to the manufacturer's recommendations (Biogal Galed Laboratories Acs Ltd.).

In peripheral blood samples, DNA was obtained using the *Ilustra blood genomic Prep Mini Spin Kit* (GE Healthcare Life Sciences, UK). For kidney tissue samples, the *Gene Jet Genomic DNA Purification* kit (Thermo Scientific, USA) was used, following the manufacturer's protocols.

Molecular detection of *E. canis* was performed by the nPCR technique, in which two pairs of initiator oligonucleotides were used according to Wen et al. (1997). Sequences of the first step of PCR reaction were "EC1 sense" AGAACGAACGCTGGCGGCAAGCC and "EC1 antisense" CGTATTACCGCGGCTGCTGGC reaction. Sequences of the second step were "EC2 sense" CAATTATTTATAGCCTCTGGCTATAGGAA and "EC2 antisense" TATAGGTACCGTCATTATCTTCCCTAT. The reaction was prepared for a total volume of 25 µL containing 0.625 U of Taq DNA Polymerase (Invitrogen, USA) enzyme, 1.5 mM $MgCl_2$, 1x Taq buffer, 0.2 mM of dNTP (Invitrogen, USA), 400 nM of primers and 15.05 µL of sterile ultra-pure water. Amplification was performed in a thermocycler (Bioer Gene pro), at 94ºC for five minutes, subsequently for 40 cycles: at 94ºC for one minute, at 60ºC for one minute and at 72ºC for one minute and a final cycle of five minutes at 72°C. Reaction cycles were similar for both steps. The only difference were the initiator

oligonucleotide pairs "EC1 sense" and "EC1 antisense" for the first round of amplification, and "EC2 sense" and "EC2 antisense" for the second round (48).

The PCR product was approximately 390 base pairs (bp) long, which was visualized by ethidium bromide staining (0.5µg/mL) and 1.5% agarose gel electrophoresis on an UV transilluminator (BioAgency).

Diagnosis of leptospirosis and VL

Microscopic Agglutination serum technique (SAM) was performed in the Laboratory of Bacterial Diseases of the Reproductive System at the São Paulo Institute of Biology for detection of anti-*Leptospira* antibodies against 24 serovars.

Serologic tests used for VL diagnosis were: DPP (immunochromatography test with rK39 antigen), and immunoenzymatic test (*Enzyme-linked immunosorbent as*say – ELISA) using total *L. major-like* antigens, both produced by the Bio-Manguinhos Biotechnology Institute. For confirmation of VL, popliteal lymph nodes and/or sternal bone marrow aspiration were obtained for direct research of *Leishmania* spp. amastigotes.

Furthermore, immunohistochemical analysis was also performed in renal tissue to exclude samples positive for *Leishmania*. Renal tissue sections were processed and submitted to endogenous peroxidase block, subsequently treated with Tris-HCl, pH 1.0 solution. Tissues were then incubated with the following primary antibodies: polyclonal mouse antibody anti-*Leishmania amazonensis* [Laboratory of Seroepidemiology and Immunobiology-IMT-USP-SP] (13). After incubation, amplification stages of the reaction occurred using the *Envision+System*-HRP*Labelled polymer, anti-mouse,* DAKO. The reaction was developed with 3,3'-diaminobenzidine (Sigma Chemical, USA) in PBS with hydrogen peroxide. Counterstaining was performed with Harry's hematoxylin.
Laboratory tests.

Five mL of peripheral blood obtained by venipuncture were placed into a tube containing ethylene diamine tetraacetic acid – EDTA (BDVacutainer) for complete blood cell (CBC) count. Blood samples were analyzed inan automated blood counter (ABC Vet, ABX Diagnostics, Montpelier, France).

Serum dosage was determined by an automated system (Thermoplate TP Analyzer) using specific commercial kits (Labtest Diagnóstica, Lagoa Santa, MG, Brazil), according to the manufacturer's instructions for quantitative measurement of total serum protein (Ref. 99-250), albumin (Ref. 19-1/250), urea (Ref. 27), creatinine (Ref. 35-100) and alanine aminotransferase (ALT) (Ref. 53-200).

Anesthetic procedure and euthanasia

Under deep venous anesthesia associated with acepromazine (2 mg/Kg) and sodium tiopenthal (25 mg/kg), the dogs were sacrificed with a 20% solution of potassium chloride. Kidney fragments were collected for histopathological exam. Fragments were maintained in 10% buffered formalin with phosphate 0.01M pH 7.2, and/or in Duboscq-Brazil fixative solution for one hour and then transferred to buffered formalin for 24 hours until processing.

Histopathological analysis

Renal tissue samples were processed by routine histological technique and stained with hematoxylin and eosin (HE). Eighty glomeruli for characterization of glomerular changes were evaluated (8).Three sections were prepared for the evaluation and classification of renal lesions. Histologic sections were also submitted to specific staining with Periodic acid-Schiff (PAS), since PAS delineates in great detail glomerular capillary basement membranes and tubular epithelial cells, the mesangial matrix and potential expansion(1);Masson's trichrome stain (MT) for the detection of collagen-based fibrous elements; periodic acid methenamine silver (PAMS), specific for basal membrane, was used for visualization of reticular fibers of the connective tissue; and Congo red stain for visualization of amyloid deposit (43). On histopathological exam, lesions were semi-quantitatively evaluated, measuring the intensity of glomerular and tubulointerstitial lesions on a scale of 0 to 4, where: 0 = normal, 1 = minimal; 2 = mild; 3 = moderate; 4 = marked (8). Glomerular diseases were classified according to Maxie and Newman (2007), as follows: 1) Membranous glomerulonephritis: basement membrane thickening predominates; 2) Proliferative (mesangioproliferative) glomerulonephritis: cellular proliferation predominates; 3) Membranoproliferative (mesangiocapillary) glomerulonephritis: both basement membrane thickening and cellular proliferation are present; and 4) Sclerotic glomerulonephritis: increased mesangial matrix combined with obliteration of capillary lumina. In this case, progressive hyalinization sometimes results in glomerular obsolescence and the glomerulus became a shrunken, eosinophilic, hypocellular mass. Glomerular location of the lesion was characterized as segmental or global. Lesion distribution was categorized as focal or diffuse (7). Glomerular cellularity and glomerular size were assessed in 30 randomly selected glomeruli on HE-stained paraffin sections (12, 42). Differential quantification of inflammatory cells in the renal cortical region was manually performed in 10 random fields in the Leica Qwin D-1000 computerized image analyzer, version 4.1 (Cambridge, UK). Data was exposed in cells/500.000 μm^2.

Quantitative results were analyzed by parametric or nonparametric tests using the GraphPad Prism 6 demo statistical software (GraphPad Software Inc., USA). Differences were considered significant when $p < 0.05$.

Results and discussion

In kidney disease of dogs with CME, pathological aspects have still not been fully elucidated (9, 10, 14, 33,) despite evidence in some studies that *E. canis* infection may cause renal lesion (16, 17, 20). Given that CME is endemic in our region and knowledge of profile of renal lesion can contribute to therapeutics, we proposed to characterize renal histopathological patterns correlated with clinical and laboratory aspects in 12 dogs naturally infected with *E. canis*. It is worth mentioning that all dogs with CME tested negative for visceral leishmaniasis and leptospirosis.

Clinical and laboratory evaluation

Clinical evaluation of 12 dogs with CME was nonspecific. Lymphadenopathy and skin lesions were the main findings, both observed in 83.33% of dogs. These signs are commonly described in different stages of canine ehrlichiosis (5, 30, 38). All twelve dogs suspected of having *E. canis* infection tested positive on serological and molecular tests.

The hematological profile of 12 dogs with CME primarily showed anemia and thrombocytopenia. Normocytic normochromic anemia and a decrease in platelets was present in 80% and 41.67% of cases, respectively. Leukopenia and leukocytosis were also observed in 25% and 33.33% of cases. In CME, hematological alterations are frequently reported and a multifactorial mechanism has been implicated (2, 6, 14, 29).

In CME, an increase in serum creatinine and urea levels has been reported (45). Based on these data, we also evaluated blood serum urea and creatinine in dogs infected with CME. Circulating levels of urea and creatinine were unaltered in these dogs (Table 1). Prerenal azotemia may occur in acute cases of ehrlichiosis due to severe dehydration (3) and renal azotemia may appear in chronic cases with severe glomerulonephritis (40). Nevertheless, the lack and/or low rate of increasing levels of these analytes into the circulation do not exclude renal lesion, since renal azotemia is detected when more than 75% of glomeruli are affected (46). In addition, the study dogs had been naturally infected. Therefore, the exact phase of infection and consequent level of kidney damage in these animals was unknown.

Hyperglobulinemia and hypoalbuminemia were also observed in our dogs, occurring in 91.67% and 41.67% of dogs, respectively (Table 1). These alterations are classically described in CME. Hyperglobulinemia in CME is caused mainly by polyclonal gammopathy. An increase in gamma globulin concentration occurs, reaching its peak in the febrile stage and persisting in the subclinical and chronic phases (35). Hypoalbuminemia in ehrlichiosis seems to result from increased vascular permeability, hemorrhage, and hepatic (49) and/or glomerular and tubular damage (9, 10).

Renal histopathology in dogs with CME and correlation with clinical and laboratory findings

It is well known that *E. canis* infection may affect different organs, including the kidneys (14, 33). However, there are few studies evaluating the renal histopathological pattern in dogs with CME, particularly characterizing glomerular diseases (9, 10, 14).

In our study, 12 dogs with CME showed glomerular and tubulointerstitial changes in 100% of the cases, exhibiting minimal to moderate lesions. The importance of microscopic examination became clear, since gross changes are minimal in some cases of the disease, as observed in the majority of animals in this study. Only one case with moderate histopathological alterations showed kidneys with a focal pale red and irregular surface that was firm due to fibrosis. Interestingly, we detected *E. canis* DNA in renal tissue of two dogs with more marked glomerular lesion and interstitial nephritis, suggesting that the presence and/or persistence of bacteria may contribute to pathological alterations in this organ (Table 2). It is noteworthy that these samples were negative for *Leishmania* detection on immunohistochemical analysis. In VL, renal lesions are classically described and associated with different types of glomerulopathies with varying degrees of severity (13).

In the chronic phase, the magnitude of clinical pathological alterations is variable and depends on host immune response and strain inoculated. A study carried out by Hasewaga (2005) in dogs experimentally infected with *E. canis* assessed disease progression for 70 days. The clinical and laboratory picture of these animals showed improvement after the 20th day of infection. However, persistent infection was confirmed by PCR positivity and splenomegaly until the 10th week after *E. canis* inoculation. In this study, no clinical signs suggestive of renal damage were cited during the acute and/or chronic phase. In our dogs, clinical signs suggestive of renal lesion, e.g. mucosal ulcerations, polydipsia and/or polyuria, as well as uremic breath were not observed. Clinical manifestations were nonspecific and unrelated to renal damage evidenced in these animals. It is noteworthy that renal tissue lesions observed in the study dogs, even in those with moderate lesions, was not necessarily representative of the entire organ and clinical signs may not develop. The clinical phase of natural *E. canis* dog infection is extremely difficult to determine. A variety of nonspecific clinical signs appear as a result of infection, which makes diagnosis difficult (31).

Table 1. Hematological and biochemical profile* of 12 dogs naturally infected with *E. canis.*

Dogs	RBC (x 10^6/mm^3)	HGB (g/dL)	HT (%)	MCV (fL)	MCHC (%)	Leuk (x 10^3/mm^3)	Platelets (x 10^3/mm^3)	Urea (mg/dL)	Creatinine (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	ALT# UI/L
1	5.11	11.9	36.5	73	31.8	5.7	124	33.00	1.50	8.03	2.94	5.09	25.80
2	5.38	13.1	35.0	65	36.0	8.3	25	59.00	1.54	8.33	3.87	4.46	37.80
3	3.49	9.5	24.2	69	33.6	5.6	46	50.00	0.76	9.00	2.84	6.16	39.30
4	5.32	12.4	33.2	61	35.5	7.7	382	52.00	0.65	7.60	1.90	5.70	12.00
5	4.12	10.1	23.9	58	32.1	3.2	111	34.00	0.88	8.85	3.44	5.41	38.76
6	6.41	14.0	44.1	69	31.7	11.2	27	23.00	1.35	8.90	1.40	7.50	23.40
7	7.83	14.6	47.9	61	32.4	8.2	364	24.40	1.00	7.40	2.60	4.80	68.49
8	4.10	11.4	27.9	67	32.1	23.5	67	20.32	1.01	8.06	3.35	4.71	26.97
9	3.97	8.4	25.8	65	32.4	33.1	273	20.00	1.25	4.10	2.60	1.50	31.80
10	5.00	7.4	23.4	59	31.7	11.7	298	14.00	0.58	8.50	2.20	6.30	36.30
11	5.31	11.0	33.5	63	32.9	20.0	470	51.00	1.02	8.80	2.10	6.70	15.00
12	4.62	9.0	27.8	60	32.3	32.4	219	26.40	0.84	7.05	1.40	5.65	10.89

*Reference values: RBC (red blood cells)= 5.5-8.5 (x10^6/mm^3);HGB (hemoglobin)= 12-18 g/dL;HT (haematocrit)= 37-55%; MCV= 60-77 fL; MCHC= 32-36 %; Leuk (leukocytes)= 6-17 x 10^3/mm^3; Platelets= 200-500 x10^3/mm^3 (24); urea: 21,40-59,92 mg/dL; creatinine: 0,50-1,50 mg/dL; Total protein: 5,40-7,10 g/dL; albumin: 2,60-3,30 g/dL; globulin: 2,70-4,40 g/dL; ALT:21-73 UI/L. # ALT= alanine aminotransferase [22, 27].

Table 2. Detection of *E. canis* in renal tissue by nPCR and histopathological analysis in kidneys of dogs with CME.

Dogs	Type and intensity of Glomerulopathy	Interstitial nephritis ♯ Mononuclear inflammatory cell infiltrate		nPCR
		Corticomedullary	Cortical	
1	Mild diffuse membranoproliferative	0	1	+
2	Moderate focal membranoproliferative	3	4	+
3	Moderate focal membranoproliferative	4	4	+
4	Mild focal membranoproliferative	3	1	-
5	Mild focal membranoproliferative	2	2	-
6	Moderate focal membranoproliferative	1	1	-
7	Mild diffuse membranoproliferative	2	4	-
8	Moderate focal membranoproliferative	1	2	+
9	Moderate focal membranoproliferative	1	1	-
10	Mild diffuse proliferative	1	2	-
11	Mild focal proliferative	0	1	-
12	Moderate focal membranoproliferative	0	1	-

♯ = Scale of lesion intensity (0 = normal, 1 = minimum, 2 = mild, 3 = moderate and 4 = marked).

Hypoalbuminemia was also a common alteration that may be indicative of renal and/or hepatic lesion (10, 47). Notably, hypoalbuminemia was negatively correlated with severity of glomerular disease in our study population, suggesting that a decrease in serum albumin level is associated with glomerular damage according to lesion intensity (Fig. 1). Hypoalbuminemia caused by liver damage apparently did not occur in these cases, since according to Thrall et al. (2007), decreased serum albumin levels due to liver disease is only observed when there is a loss of 60 to 80% of liver function. In these cases, ascitis normally occurs. However, these animals did not show ascites in both the clinical examination and autopsy.

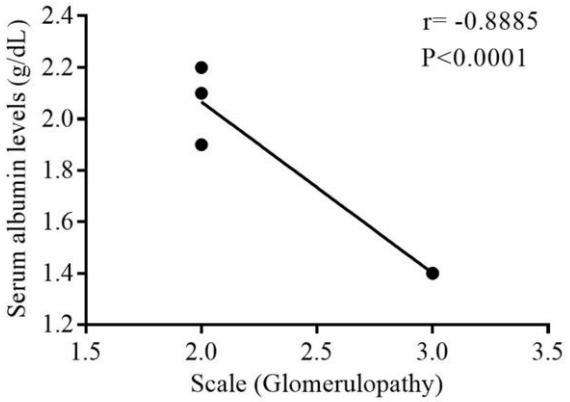

Figure 1. Negative correlation between serum albumin levels and severity of glomerulopathy in dogs naturally infected with *E. canis*. Spearman's test.

In this study, dogs with CME did not have altered serum levels of urea and creatinine. Therefore, a correlation between analyte levels and histopathologic renal findings was not evidenced in these dogs. It is noteworthy that more than 70% of the kidneys need to be involved for serum detection of these analytes (46).

Studies with dogs naturally infected with *E. canis* failed to observe glomerular lesions (20). However, in experimental infections minimal glomerular and tubular lesions have been described (9, 10, 14). Thus, we highlight the importance of our research study, the first to demonstrate that natural *E. canis* infection in dogs may cause significant glomerular damage. We confirmed that 50% of dogs with CME had moderate glomerulopathy accompanied by a minimal to moderate interstitial nephritis (Table 2).

Glomerular alterations in dogs with CME

To better understand the histopathological renal findings in dogs with CME, we characterized glomerulopathies according to the classification described by Maxie and Newman (26), as follows: membranous, proliferative, membranoproliferative and sclerotic. In addition, we evaluated the distribution of glomerular lesions. It is worth mentioning that we failed to find any information in the literature pertaining to this approach in dogs naturally infected with *E. canis*.

Glomerulopathy was present in 100% of infected animals. The distribution of glomerular lesion was focal and diffuse in 75% and 25% of cases, respectively (Table 3). Membranoproliferative glomerulopathy predominated in 83.33% (10/12) of dogs with CME. In the glomerulus, we observed a striking increase in mononuclear cells in the mesangium accompanied by thickening of the mesangial matrix and glomerular capillary basement membrane, confirmed by PAS staining (Fig. 3). Furthermore, we observed an increase in the glomerular tuft and a decrease in the urinary space, particularly in moderate lesions. Glomerular hypercellularity was confirmed by the number of cells counted in 30 glomeruli. Dogs infected with *E. canis* had higher glomerular cellularity compared to noninfected control dogs (Fig. 2). Capillary damage was absent and/or minimal. Other glomerular injuries of lower intensity were: protein deposits in the urinary space, thickening of the urinary capsule, and mild glomerular sclerosis (Fig. 4). Proliferative glomerulopathy of mild intensity was present in only two dogs (Fig. 4). Both cases showed a global lesion, whose distribution was diffuse in one case and focal in the other. In this type of glomerular lesion, we observed an increased number of mononuclear cells in the mesangium and expansion of the mesangial matrix. Membranoproliferative glomerulonephritis has a poor prognosis and progresses to end-stage renal failure (4).

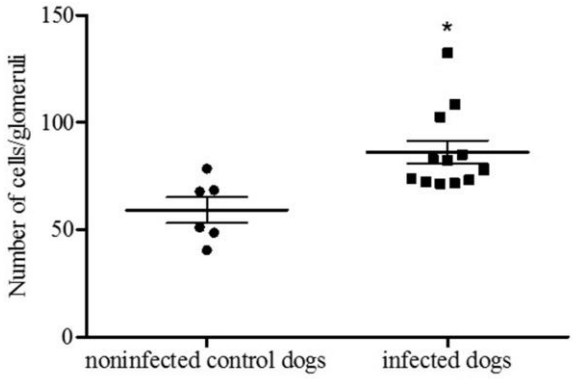

Figure 2. Glomerular cellularity. Number of cells per glomerulus is higher in kidneys of dogs infected with *E. canis* when compared with noninfected control dogs. *p<0.05, Mann-Whitney test.

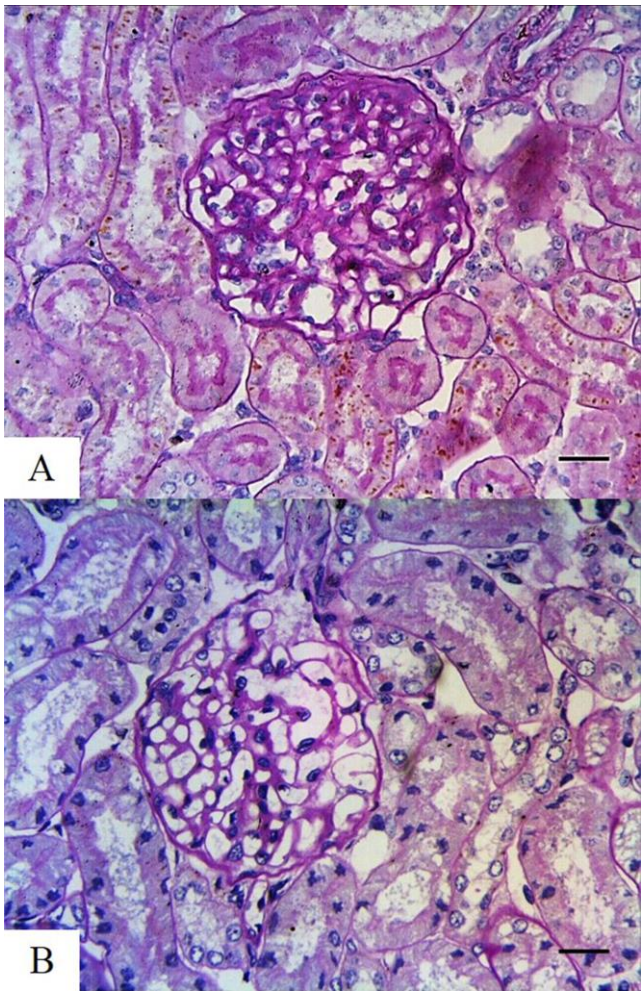

Figure 3. Membranoproliferative glomerulopathy in dogs naturally infected with *E. canis*. (A) Glomerular capillary basement membrane and mesangial matrix thickness; (B) Glomerulus without glomerular capillary basement membrane thickness. PAMS. Bar = 25 µm.

In chronic kidney disease, renal injury is associated with a direct or indirect action of the immune system. In general, a lesion directly mediated by the immune system affects the glomerulus causing different profiles of glomerulonephritis (37). Based on our data, *E. canis* infection seems to induce a particular type of membranoproliferative glomerular lesion. In studies evaluating renal lesion in dogs infected with *E. canis* either naturally and/or experimentally, the intensity of any alteration was minimal and the type of glomerulonephritis was not characterized (9, 10, 15).

Interstitial alterations in dogs with CME

In addition to glomerular alterations, we also observed tubulointerstitial lesions in all dogs with CME. Interstitial nephritis predominated in 100% of cases, with intensity ranging from minimal to marked. Inflammatory infiltrates occurred more intensely in the cortex of the interstitial, perivascular and/or periglomerular regions (Table 4; Fig. 5). Codner et al. (10) observed lymphoplasmacytic infiltrate especially in the cortical region. Another study conducted in dogs naturally infected with *E. canis* demonstrated that 98% of animals exhibited plasma cell infiltration surrounding veins and arteries of the corticomedular region (20). Plasma cell and histiocytic infiltrates were also present in the perivascular cortical region, extending to the corticomedullary region in the acute phase of experimental infection (33).

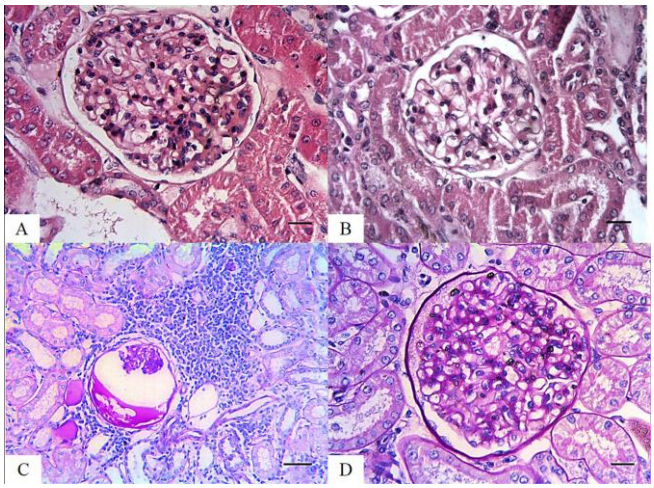

Figure 4. Glomerular lesion in kidney of dogs naturally infected with *E. canis*. (A) PGN. Glomerular hypercellularity (H&E). Bar = 25 µm. (B) Normal glomerulus (H&E). Bar = 25 µm. (C) Protein deposits in the urinary space (arrow) and glomerular sclerosis (arrowhead) (PAS). Bar= 50 µm. (D) PGN. Reduced of urinary space and mesangial matrix thickness (PAS). Bar = 25 µm.

The pathogenesis of CME appears to be related to antibody-dependent cytotoxicity (14, 21). According to Hildebrandt e al. (20), this leads to intense vasculitis in different organs, where perivascular plasma cell inflammatory infiltration predominates. In our analysis, we observed a perivascular lymphohistioplasmacytic infiltrate in the cortical region of kidney without evidence of vessel wall damage, suggesting that other mechanisms may be involved in the development of CME renal lesions.

In the cortical region, inflammatory infiltrates were mainly lymphohistioplasmacytic in 58.33% of cases, where there was a significant predominance of lymphocytes compared to plasma cells and histiocytes on differential tissue count (Fig. 6). In experimental infection induced by Codner et al. (10), cell infiltrates mainly composed of lymphocytes and plasma cells predominated in the cortical region. A perivascular lympho-histioplasmacytic infiltrate was also observed in the corticomedullary region in 33.33% of dogs, similar to findings obtained by other studies on natural infection

(20). In the pathophysiology of glomerulonephritis, glomerular lesion may alter homeostasis in the tubulointerstitial region through diverse mechanisms. One mechanism is T-cell recruitment, resulting from the production of inflammatory cytokines and chemotactic factors by kidney-resident dendritic cells (23). Since the disease causes immunoregulation, it may be inferred that T lymphocyte predominance contributes to the pathogenesis of interstitial nephritis in CME.

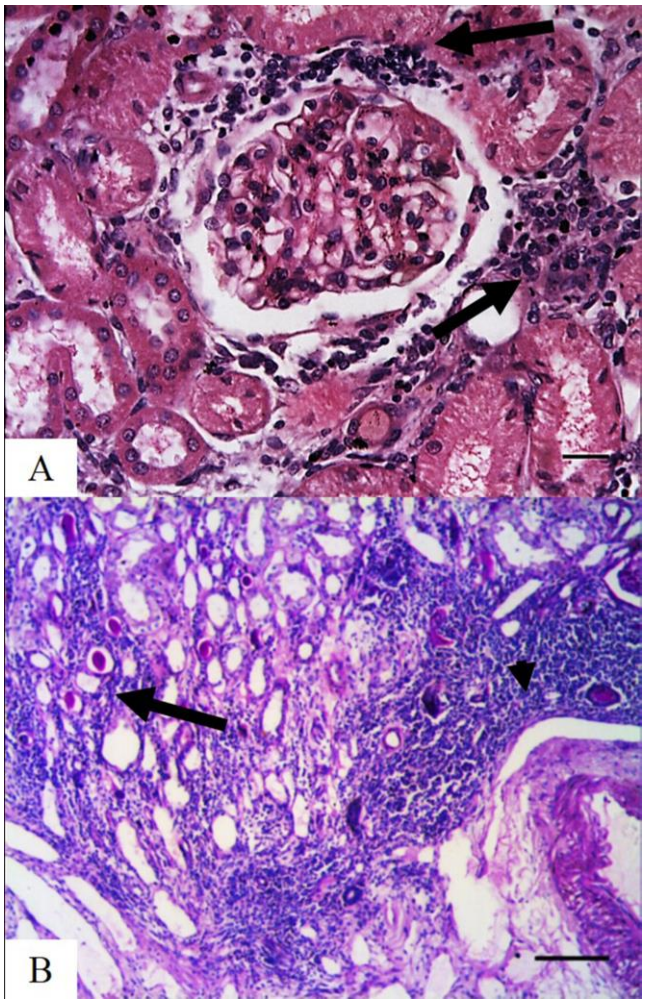

Figure 5. Inflammatory infiltrate in kidney of dogs naturally infected with *E. canis*. (A) Periglomerular mononuclear inflammatory infiltrate (arrows). H&E. Bar = 25 µm. (B) Interstitial (arrow) and perivascular (arrowhead) mononuclear inflammatory infiltrate. H&E. Bar = 100 µm.

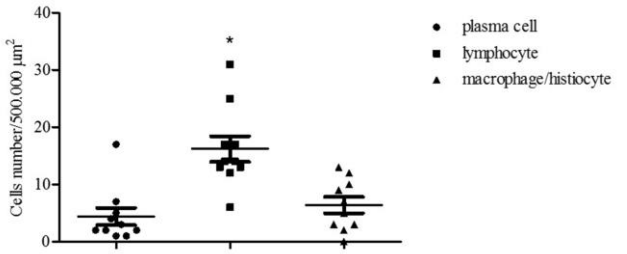

Figure 6. Differential tissue count of inflammatory infiltrate in kidney of dogs naturally infected with *E. canis*. Significant predominance of lymphocytes in inflammatory infiltrate in cortical area. *p< 0.05, Kruskal-Wallis and Dunns tests.

Minimal or moderate interstitial fibrosis was present in 33.33% of dogs in this study (Table 4). Fibrosis is not a lesion commonly described in CME, probably because analyses were conducted in the acute or subclinical phase of the disease (9, 10, 14, 20, 32). It is widely known that renal injury produced by *E. canis* occurs more commonly in the chronic phase (15). In our findings, fibrosis was more evident in cases with more intense inflammatory cell infiltration. Only one animal showed moderate fibrosis (Fig. 7), which was associated with multifocal to coalescing infiltration of inflammatory cells. In this case, there was tubular dilatation or atrophy in areas exhibiting more intense fibrosis. Moderate membrane proliferative glomerulopathy was also observed. However, the animal did not show any alterations in urea and creatinine serum levels, probably because fibrosis failed to affect the full length of the kidney.

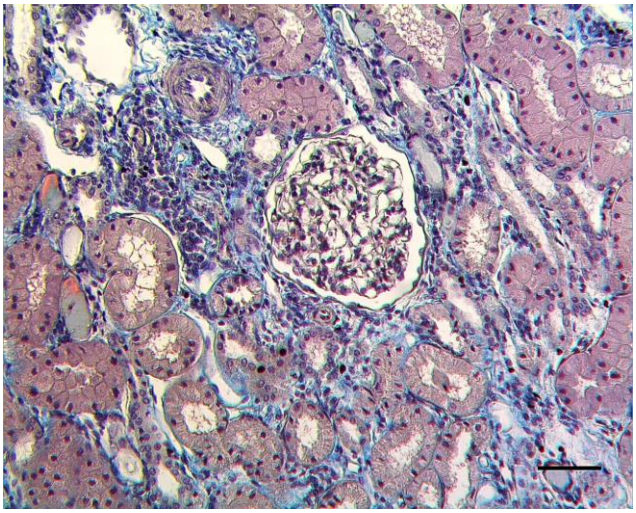

Figure 7. Interstitial fibrosis in kidney of dogs naturally infected with *E. canis*. Presence of moderate interstitial fibrosis in the peritubular and periglomerular region. Masson's trichrome. Bar = 50 µm.

Table 3. Glomerular alterations observed in kidneys of dogs naturally infected with *E. canis.*

Dogs	Glomerulophaty		Glom hypercell♯	Glomerular Sclerosis	Mesang thick¥	Urinary capsule thick£	Urinary space thick∞	Protein dep urinary spaceΩ	Reduced BM space α	GM lob β
	Type	Intensity								
1	MPGN	2*	4	1	2	4	2	0	4	1
2	MPGN	3	3	2	1	0	0	0	1	0
3	MPGN	3	2	1	3	3	3	3	3	3
4	MPGN	2	4	0	3	2	4	1	4	0
5	MPGN	2	3	1	3	2	3	0	4	2
6	MPGN	2	3	2	3	1	4	0	4	0
7	MPGN	3	2	0	0	0	2	0	2	0
8	MPGN	3	3	2	3	1	2	0	2	1
9	MPGN	3	2	2	2	2	2	0	1	3
10	PGN	2	3	2	3	1	3	0	1	0
11	PGN	2	3	0	3	1	3	0	3	3
12	MPGN	3	4	0	3	2	2	0	2	3

MPGN= membranoproliferative glomerulonephritis. PGN= proliferative glomerulonephritis. * scale of lesion intensity (0 = normal, 1 = minimal, 2 = mild, 3 = moderate and 4 = marked). # = glomerular hypercellularity; ¥ = mesangial matrix thickness; £ = glomerular capillary basement membrane thickness; ∞ = Urinary capsule thickness; Ω = protein deposits in the urinary space; α = reduced urinary space; β = glomerular lobulation.

Table 4. Histological characteristics of interstitial nephritis and other lesions observed in kidney of dogs naturally infected with *E. canis*.

Dog	Interstitial nephritis			Other lesions
	Inflammatory infiltrate type	Distribution	Intensity	
1	Lymphohistioplasmacytic	Interstitial, perivascular, periglomerular focal	Minimum	Tubular vacuolar degeneration, necrosis, dilatation and atrophy; hyaline casts
2	Lymphohistioplasmacytic	Interstitial, perivascular, periglomerular multifocal tocoalescing	Marked	Tubular vacuolar degeneration, necrosis, dilatation and atrophy; hyaline casts; interstitial fibrosis
3	Lymphoplasmahistiocytic	Interstitial, perivascular, periglomerular, subcapsular multifocal tocoalescing	Marked	Tubular vacuolar degeneration, necrosis, dilatation and atrophy; hyaline casts; interstitial fibrosis
4	Lymphohistioplasmacytic	Interstitial, perivascular, periglomerular Multifocal	Mild	Interstitial fibrosis
5	Lymphohistioplasmacytic	Interstitial, perivascular multifocal	Minimum	Tubular vacuolar degeneration
6	Lymphohistioplasmacytic	Interstitial, perivascular multifocal	Minimum	Tubular vacuolar degeneration, necrosis and atrophy; hyaline casts
7	Plasmalymphocytic	Interstitial focal	Marked	Hyaline casts
8	Lymphohistioplasmacytic	Interstitial, perivascular, subcapsular Multifocal	Mild	Tubular vacuolar degeneration; hyaline casts; interstitial fibrosis
9	Lymphohistioplasmacytic	Interstitial, perivascular, periglomerular multifocal	Minimum	Tubular vacuolar degeneration
10	Lymphoplasmacytic	Interstitial, perivascular, periglomerular multifocal	Mild	Tubular vacuolar degeneration and dilatation
11	Histiolymphocytic	Interstitial focal	Minimum	Tubular vacuolar degeneration, necrosis and dilatation
12	Histioplasmalymphocytic	Interstitial focal	Minimum	None

Renal hyaline casts were found in 50% of our dogs. In two cases, hyaline casts of marked intensity were present (Table 4; Fig. 8). Urinalysis was not possible in this study, but this finding confirmed proteinuria in these animals. According to Troy and Forrester (44) proteinuria, with or without azotemia, may occur in dogs with ehrlichiosis. In the acute phase of CME, it was observed that proteinuria was inversely correlated with serum albumin levels, suggesting that hypoalbuminemia in CME may be due to protein loss from the kidneys (10).

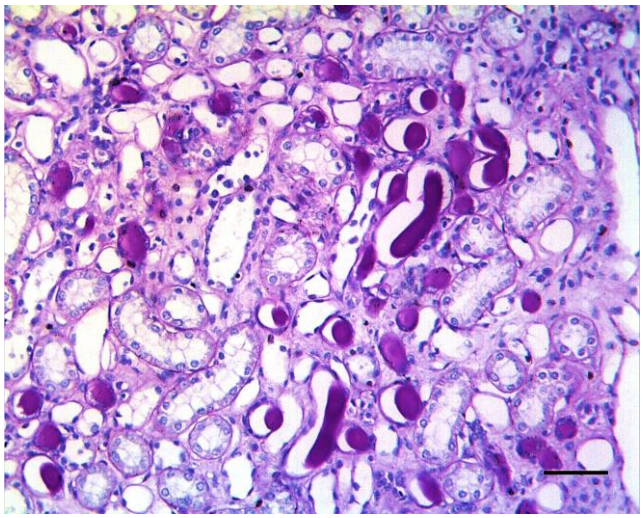

Figure 8. Presence of hyaline casts in kidneys of dogs naturally infected with *E. canis*. Hyaline casts in renal tubules (arrows). PAS. Bar = 50 μm.

Circulatory changes including vascular congestion, hemorrhage and edema were also occasionally observed. Furthermore, in the medullary region there were two cases of lymphocytic pyelitis ranging in intensity from minimal to mild, and four cases of pyelonephritis, with mild to marked intensity.

Conclusions

This study provides first data on the characterization of glomerulopathy in renal lesions of dogs naturally infected with *E. canis*. Our findings showed that membranoproliferative glomerulonephritis was the main glomerular lesion associated with infection by this bacterium. Although we did not find any cases with clinical manifestations due to renal damage, our data demonstrate the importance of histological renal findings associated with protein loss before the occurrence of clinical disease. Thus, mild glomerular changes seem to suffice for hypoalbuminemia even in cases of focal lesions. The pathogenesis of membranoproliferative glomerulonephritis in dogs is often associated with immunocomplex deposition. However, interstitial nephritis associated with lymphocyte infiltrate suggests that these cells may also play an important role in the immunopathogenesis of renal lesion in dogs with CME, as shown in this study. Therefore, further studies to clarify the involvement of these cells in the pathogenesis of nephropathy associated with infection by *E. canis* in dogs are needed to provide more accurate information for the diagnosis, treatment and prognosis of CME.

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