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Renal Lesions Associated to *Leptospira* spp. seroprevalence in Pigs at Portoviejo Slaughterhouse

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ABSTRACT

Background: Leptospirosis has been little studied in Ecuador, where it is highly prevalent in the coastal provinces. **Aim.** To identify histopathological patterns in the kidney, and their association with *Leptospira* spp. seroprevalence in pigs at the municipal slaughterhouse in Portoviejo canton. **Methods:** The histological sections of 420 kidneys from slaughtered pigs were studied, which demonstrated an association between renal lesions and *Leptospira* seroprevalence. Comparison of the proportion tests were conducted, and risk analysis (EPIDAT, version 3.1), for epidemiological analysis of tabulated data was performed. **Results:** The presence of serovars Australis, Bratislava, Icterohaemorrhagiae, and Canicola was evidenced in seroprevalence slaughter pigs. These factors may pose a potentially latent threat to susceptible humans, corroborating the high percentage of lesions caused by *Leptospira* spp., found in the kidneys of these animals. The evidence found represents a significant risk sending a warning to workers that handle and work with pigs sacrificed in slaughterhouses. **Conclusions:** Glomerulonephritis, tubular nephritis, interstitial nephritis, and glomerulitis appear in the kidneys of asymptomatic pigs, which test positive or not to *Leptospira* spp, with a broad spectrum of lesions caused by the infection in these animals. Pigs with apparent interstitial nephritis were 3.5-fold more likely to test positive on MAT than the ones that showed no lesion.

Key words: antibodies, animal diseases, serology, tissues, transmission (*Source: BVS*)

Citation (APA)

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INTRODUCTION

Histopathological studies conducted in pigs that tested positive to *Leptospira* by MAT (microagglutination test), and corroborated by other diagnostic methods, such as microbiological isolation and immunofluorescence, indirect immunohistochemistry, and conventional PCR (polymerase chain reaction), show inaccurate and contradictory results (Boqvist *et al.* 2003; Radaelli *et al.* 2009).

Research done in slaughter pigs showed that interstitial nephritis not always corresponds to the presence of *Leptospira* spp. (Boqvist *et al.* 2003, Martínez *et al.* 2006), and multifocal interstitial nephritis in pigs has been associated with several infectious agents (Martínez *et al.* 2006), the pathomorphological characteristics of this lesion show no uniformity, according to studies done in several countries, as a result of records of various histological patterns of interstitial nephritis in swine leptospirosis (Pezzolato *et al.* 2012).

The purpose of this study was to identify the histopathological patterns in the kidney, and their association with seroprevalence of *Leptospira* spp. in seemingly healthy pigs at the municipal slaughterhouse in Portoviejo canton.

MATERIALS AND METHODS

Location and duration: This study was conducted in Portoviejo canton (capital of Manabi province), located on 80°29,296'O west longitude; 0°53,864'S, south latitude, 18 m above sea level, in Ecuador. It took place in the period between December 2018 and March 2019 (rainy season), and May-August 2019 (dry season).

Animals: Hogs were sacrificed at the municipal slaughterhouse in Portoviejo canton, province of Manabi, and from other two nearby provinces (Santa Elena and Santo Domingo de los Tsachilas).

Sacrifice was performed according to the standards included in the Law of animal Health of AGROCALIDAD (Ecuadoran Agency for Agro Quality), and the Sanitary Code for Ground-Dwelling Animals, Chapter 7: Law of Animal Welfare of the International Office of Epizootics. The animals were chosen at random (simple randomized sampling), with no evidence of symptoms, and phenotypical characteristics from the native Ecuadoran hogs and commercial animals.

Sampling: Randomized sampling was performed (simple randomization) of blood and kidneys; the total population of animals ready for sacrifice was estimated in 1 200 pigs slaughtered on a monthly average, for a six-month period, accounting for 7 200 pigs to be sacrificed. The size of the sample was determined using the formula for finite populations, according to the expected prevalence (Thrusfield, 2018). Though the result was $n = 173$, the total number of samples was 200.

Blood sample collection for MAT: A total of 200 blood samples were collected at slaughter by sectioning the vessels around the neck, and stored in sterile 10 cm long x 1.4 cm diameter plastic tubes (ITC), without anticoagulant. The samples collected were kept at 20 °C. Following the formation of fibrin clots, the blood serum was extracted by centrifugation at 453 centrifugal force, for 10 minutes in a centrifuge (MedicLife, model 800 B, China). The serum was distributed in 2 mL aliquots, and stored at -20 °C until processing.

Blood sample collection from kidneys for histopathology: Upon slaughtering, the kidneys were checked in order to take histopathological samples, one per animal (420 samples from the total animals in the study); a number of 200 samples had been drawn from the same animals for the serological study. The renal samples were collected by 1cm² x 0.5 cm deep sectioning, using a No. 23 scalpel, and complying with the required antiseptic standards (a single set of sterile gloves and scalpel per animal). The histopathological samples were stored in 10% formol (Abreu *et al.*, 2017), in 20 mL sterile flat-bottomed plastic tubes with a screw cap, in 1:3 proportion (sample/formol), and were kept at room temperature until processing.

Methodology used for serological diagnostic: The serological diagnostic was made in the laboratory of AGROCALIDAD in Tumbaco, by microscopic agglutination (MAT), considered the reference test for serological diagnostic of leptospirosis, as recommended by OIE (2014b). Pig screening for slaughtering included a panel of eight serovarieties of *Leptospira* with representative antigens of known serogroups circulating in the region, as recommended by OIE (2018), which had been established in Ecuador by AGROCALIDAD: *L. borgpetersenii* Tarassovi, *L. interrogans* Canicola, *L. interrogans* Icterohaemorrhagiae, *L. interrogans* Australis, *L. interrogans* Bratislava, *L. interrogans* Pomona, *L. interrogans* Pyrogenes, *L. interrogans* Grippotyphosa. The panel of serovars included strains certified by the National Service of Veterinary Laboratories, at the Department of Agriculture of The United States, originally from the OIE Reference Laboratory, located in Argentina. The microbial strains were plated every week in culture media and conditions recommended by WHO (2008), in order to produce a culture in four to seven days.

The serological titers in the highest dilution were considered positive, with 50% leptospira agglutinated vs 50% free leptospira, comparing the field of study with strain control (100% free leptospira). The initial dilution was 1:50, from which serial dilutions were made (base logarithm 2), of 1:100, 1: 200, 1: 400, 1: 800, 1: 1600, 1: 3200, 1: 6400. Equal or greater titers than 100 against one or more serovars of *Leptospira* were considered seropositive, as recommended by OIE (2014b). When the same sample showed agglutination with two or more serovarieties over the same titer, it was considered a cross reaction, reported when there were titers ≥ 100 .

Methodology used for histopathological diagnostic: The kidney samples collected according to the procedure described, from 420 slaughtered pigs, were stored in separate vials (10 mL tubes with screw caps containing 10% formol), at room temperature (28 °C), until processing. Then they were dehydrated and placed in paraffin blocks, sectioned with a microtome (5 μ m), and the

tissue sections were stained with hematoxylin and eosin. The plates were observed through the microscope (Olympus CX30, 40x magnification).

Statistical processing: A database was created with Microsoft Excel, and the results were processed using STATGRAPHICS CENTURION ver. XV. II. Descriptive statigraphs were employed as absolute and relative frequencies in the seroprevalence of antibodies against *Leptospira* spp., and histopathological lesions in the kidneys. Binomial and multiple comparison of proportion tests were performed to lesions observed in pigs that tested positive and negative to *Leptospira* by MAT, and to compare seropositivity of each serovar to the mean proportion, respectively. Besides, Chi-square was used to evaluate the association between renal lesions and positivity to *Leptospira*.

A risk analysis was performed to determine the association between interstitial nephritis and *Leptospira* seroprevalence, in which a transversal analytic observational study was conducted by creating 2 x 2 contingency tables, using the program for epidemiological analysis of tabulated data (EPIDAT, version 3.1).

RESULTS AND DISCUSSION

Seroprevalence of antibodies against *Leptospira* spp. in pigs for slaughter

Of the total serological leptospirosis-diagnosed pigs sacrificed 33/200 (16.5%) CI: 95% [11.35-21.64] tested seropositive; the eight serovarieties analyzed were seropositive as well, which proves intermediate seroprevalence in these animals.

Table 1 shows that the most frequently observed serovars were Canicola 6.5% (13/200), and Pyrogenes 6% (12/200), but no significant differences between each serovar and the mean proportion were found (3.81%).

Table 1. *Leptospira* serovars present in slaughter pig sera in Portoviejo canton, in 2018-2019

Serovars	Samples	Antibody titers					Percentage
		100	200	400	800	1600	
Australis	9	6	1	1	1	0	4.5
Bratislava	6	2	4	0	0	0	3
Tarassovi	8	3	4	0	0	1	4
Pomona	4	3	1	0	0	0	2
Pyrogenes	12	11	1	0	0	0	6
Icterohaemorrhagiae	8	7	1	0	0	0	4
Canicola	13	7	5	0	1	0	6.5
Grippotyphosa	1	0	1	0	0	0	0.5
Total	61*	39	18	1	2	1	-

*Of the 33 seropositive samples resulting from cross reaction against some serovars, the number of seroreactions increased to 61.

The presence of serovars Australis, Bratislava, Icterohaemorrhagiae, and Canicola was demonstrated to circulate in slaughter pigs in Portoviejo canton. These factors can pose a

potential latent danger to susceptible humans. The inclusion of serovars like Hardjo, Bratislava, Sejroe, and Grippotyphosa in serological screenings in swine could contribute to better understanding the role of this species as reservoirs of *Leptospira* in the area where they are studied (Rodríguez *et al.* 2017).

Pedersen *et al.* (2017), in a study done in slaughter pigs, found antibodies against several demonstrated zoonotic pathogens. They did not find significant differences as to the prevalence of pathogens in terms of age, sex, or establishment; serovars Bratislava and Pomona were the most commonly found, along with serotypes with the highest titers, indicating an active infection. These results differ from the findings in this study, since the greatest percentage of seropositives was observed against serovars Canicola and Pyrogenes.

Histopathological findings in slaughter pig kidneys

The main histopathological changes observed in the kidneys of seemingly healthy pigs (Figure 1), were related to circulatory and degenerative disorders, inflammatory processes, and others. Severe nephritis was absent in all cases. In general terms, inflammation did not affect all the kidney, but particularly the glomerulus, tubules, and surrounding tissues (tubulointerstitial tissue).

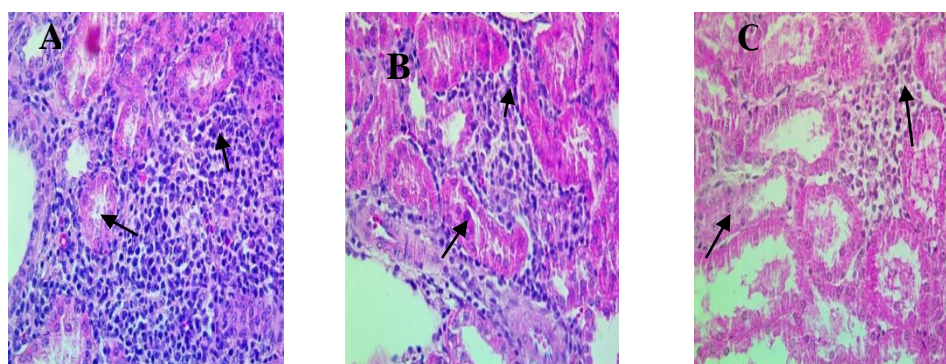


Figure 1. Renal lesions in pigs positive to *Leptospira* spp., by MAT, histological section
(A) Interstitial nephritis with inflammatory infiltrate abundant in lymphocytes and macrophages limited to perivascular areas. (B-C) Tubulointerstitial nephritis, presence of lymphoreticular cells spreading to other areas of the cortical parenchyma. H-E 40x

The seroprevalence detected in slaughter pigs corroborates the high percentage of lesions caused by *Leptospira* spp. in the kidneys of these animals. This evidence creates a significant alert on the risk the personnel that handle and work with slaughtered pigs in the facility are exposed to. The results observed matches the criteria of other authors who refer to pigs as a potential risk of infection to humans and other domestic animals, especially those living near swine farms (Benacer, Thong, and Sourist, 2017).

This disease especially affects different risk groups, such as fishermen, miners and sewage workers, garbage collectors, rice farmers, slaughterhouse workers (Gaitán, Espinosa, and

Rodríguez, 2018), and other risk groups that perform their work in highly humid environments in which pathogenic transmission is favored (Meny *et al.*, 2019).

The histopathological lesions found in *Leptospira* positive kidney sections, using MAT, match the criteria of Yang (2017, who noted that chronic renal infection caused by *Leptospira* can appear together with characteristic chronic tubulointerstitial nephritis, and interstitial nephritis. In pigs positive by MAT, the dominating lesions were multifocal interstitial nephritis, glomerulitis, and focal interstitial nephritis (Figure 2).

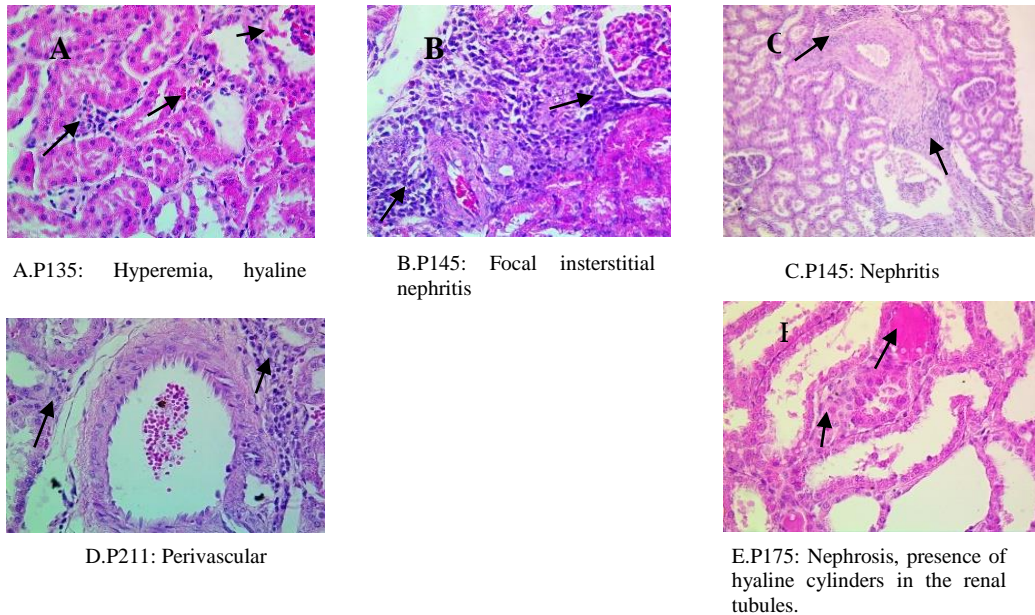


Figure 2. Histopathological lesions in *Leptospira* positive kidney segments by MAT

Observation of histological segments demonstrated that the 420 (100%) samples observed had, at least, a renal lesion detectable by optic microscopy, though not all of them were associated with *Leptospira* (glomerulitis, lipidosis, hyperemia, nephrosclerosis, tubular atrophy, and cystic nephrosis), and might be caused by other processes.

The lesions could be identified as ten different histopathological changes: glomerulitis, interstitial nephritis, nephrosis, tubular nephritis, glomerulonephritis, and in a lesser degree, lipidosis, hyperemia, nephrosclerosis, tubular atrophy, and cystic nephrosis (Table 2).

Table 2. Frequency of histopathological kidney lesions

Type of disorder	N°	%	CI: 95%
Circulatory disorders			
Hyperemia	3	0.71	-0.09-1.51
Degenerative disorders			
Tubular atrophy	10	2.38	0.92-3.84
Nephrosis	18	4.29	2.35-6.23
Nephrosclerosis	5	1.19	0.15-2.23
Inflammation			
Glomerulitis	105	25.00	20.86-29.14

Interstitial nephritis	145	34.52	29.97-39.07
Tubular nephritis	80	19.05	15.29-22.81
Cystic nephritis	1	0.24	-0.23-0.71
Glomerulonephritis	46	10.95	7.96-13.94
Other findings			
Lipidosis	7	1.67	0.44-2.90
Total	420	100	

The percentage of every lesion is increasing, and occurs in the following order: glomerulonephritis, tubular nephritis, glomerulitis, interstitial nephritis, which suggests a progress in the infectious process that goes through the early, active, and chronic phases, except for glomerulitis, which shows less tissue damage. Interstitial nephritis revealed a lymphohistiocytic lesion pattern characterized by tubulointerstitial nephritis, the presence of inflammatory infiltrate, and degeneration of tubular tissue in positive samples of leptospirosis, by MAT.

These results are similar to reports of Pan and Yang (2019), who found that *Leptospiral* endotoxins trigger immunological responses in the host, and lead to tubulointerstitial nephritis, the most commonly found histological event in animals with leptospirosis. Persistent chronic *Leptospiral* infection can cause tubulointerstitial nephritis, and will eventually develop into tubulointerstitial fibrosis in animals with chronic leptospirosis.

***Leptospira* spp. seroprevalence and its association with slaughter pig kidney lesions**

From a total of 33 seropositive pigs (100 titers) by MAT, 16 (48.48%) showed interstitial nephritis. Besides, a statistically significant association was found between this lesion and *Leptospira* seropositivity. The remaining inflammatory lesions observed showed no association with seropositivity to *Leptospira* (Table 3).

Table 3. Association between renal lesions and leptospira seropositivity in slaughter pigs, in Portoviejo canton (2018-2019)

Anatomopathological lesions	Leptospirosis		χ^2	p-value
	% Positive	% Positive		
Glomerulitis	30.30 (10/33)	38.92 (65/167)	0.87	0.35
Interstitial nephritis	48.48 (16/33)	20.95 (35/167)	10.99	0.00
Nephrosis	6.06 (2/33)	5.98 (10/167)	0.00	0.98
Tubular nephritis	42.42 (14/33)	43.11 (72/167)	0.00	0.94
Glomerulonephritis	18.18 (6/33)	22.15 (37/167)	0.25	0.61

P ≤ 0.01 indicate significant differences, according to the χ^2 test.

Interstitial nephritis is a frequent cause of pork condemnation in slaughterhouses (Lazo *et al.*, 2017; Ortiz *et al.*, 2018; Ortiz *et al.*, 2019), and several researchers relate it to infection by leptospira and other bacterial, and viral agents (Boqvist *et al.*, 2003; Martínez *et al.*, 2006; Radaelli *et al.*, 2009; Pezzolato *et al.*, 2012; Strutzberg *et al.*, 2018; Yang, Pan, and Yang, 2019; Carrillo *et al.*, 2019). However, in pigs, the results are ambiguous, since interstitial or multifocal

interstitial nephritis have not always been corroborated in the presence of *Leptospira* spp. The inflammatory patterns found are multiple, and studies that demonstrate the interrelation between the identification of *Leptospira* spp. by serological methods (MAT) and histopathological patterns of renal lesions caused by infection, are few.

Another type of analysis that clearly shows the association between interstitial nephritis and *Leptospira* seropositivity, is risk analysis (Table 4). It shows that out of 51 pigs whose kidneys suffered interstitial nephritis, 16 (31.37%) tested positive to leptospirosis by MAT, as opposed to 17 out of 149 (only 11.40% positive by MAT), in pigs whose kidneys did not have that lesion. The pigs with interstitial nephritis were 3.5-fold more likely to test positive by MAT than the ones that showed no lesion. Thus, the association between interstitial nephritis and positivity by MAT was evident (OR = 3.54). This association is significant CI: 95 % (1.63 – 7.72), and statistically $p \leq 0.001$.

Table 4. Risk analysis and association between interstitial nephritis and *Leptospira* seropositivity, by MAT.

Statistical significance							
Variables	Level of Exposure	Number of positive samples/Total	Frequency (%)	OR	CI 95%	χ^2	P value
Interstitial nephritis	Yes	16/51	31.37	3.54	1.63–7.72	10.99	0.00
	No	17/149	11.40				

OR: odds ratio

These results demonstrate that seemingly healthy pigs at the slaughterhouse, whose *postmortem* inspection shows the occurrence of interstitial nephritis, are more likely to have been infected by *Leptospira*. Therefore, ordered and systematic recording of condemnations can assist early epidemiological alert and surveillance systems, which through proper traceability and feedback from farms, can contribute to the prevention and control of swine leptospirosis in the territory.

Moreover, corroboration of the microbiological diagnostic of leptospirosis by bacterial isolation is difficult and painstaking. Serological diagnostic by MAT requires uncontaminated live cells, and it is painstaking as well. In veterinary diagnostic laboratories, the finding of interstitial nephritis in the macro and micro anatomopathological examination, along with data from anamnesis, clinical exploration, epidemiological diagnostic, and other complementary examinations, help corroborate a presumptive diagnostic of leptospirosis, additionally, they provide early alert, and facilitate preventive and control measures on infected farms.

These results are similar to Baker *et al.* (1989), who conducted a survey in the slaughterhouse in order to determine the prevalence of leptospirosis, and its association with multifocal interstitial nephritis lesions in pigs at sacrifice, and found a significant association ($p = 0.046$), and a strong association (OR = 8.10) between multifocal interstitial nephritis (MFIN) and the presence of renal leptospiras detected by culturing, in addition to an association between leptospira titers and

MFN lesions in the group of animals surveyed for prevalence, which was statistically significant ($p = 0.031$), but differed from the results of Boqvist *et al.* (2003), who studied 32 fattening pigs in a slaughterhouse in the south of Vietnam, but found no association between the presence of leptospires and nephritis ($p = 0.19$).

Table 5 shows morphopathological lesions in *Leptospira* positive and negative pigs, following diagnostic tests. The predominant lesions found in positive pigs by MAT corresponded to glomerulonephritis, tubular nephritis, and interstitial nephritis, accounting for 55.10% (27/49), contrary to the behavior observed in negative pigs by MAT, where glomerulitis was the significant lesion, accounting for 31.91% (15/47). This result differs significantly for $p \leq 0.05$.

Table 5. Morphopathological lesions in *Leptospira* positive and negative pigs by MAT

Lesions	N	No.	Percentage	CI: 95 %	Results of diagnostic test
Glomerulonephritis, tubulointerstitial nephritis	49	27	55.10 ^a	41.17-69.03	MAT +
Glomerulitis	47	15	31.91 ^b	18.58-45.24	MAT -

Legend: Different superscripts on the same column indicate significant differences $p \leq 0.05$. Binomial comparison of proportions

In this study, the identification of predominant morphological patterns, based on MAT results and histopathology revealed that glomerulonephritis and tubulointerstitial nephritis are associated with *Leptospira* seropositivity. Furthermore, glomerulitis has been found to be associated with other possible infections on noninfectious pathological processes.

In cases where infectious are incipient, and the absence of antibodies against *Leptospira*, higher than one titer out of 100, glomerulonephritis stood out as the lesion pattern, accounting for 68.18% (15/22), with 50 titers (seroreacting), which showed that lesion, too. During this phase of *Leptospira* infection, a slight renal pathological response develops without a significant presence of other lesions. This is explained by the absence of any microscopic change in the presence of renal colonization by *Leptospira*, which is likely to happen when the arrival of the bacterium at the renal epithelium takes place at such an early stage that it cannot cause proper stimulation of local cellular immune response (Monahan, Callanan and Nally, 2009).

In active cases with the presence of antibodies against *Leptospira*, higher than one titer out of 100, the pattern of the lesion was characterized by tubular nephritis. Fifty percent (4/8) showed corresponding titers of 100 and 200, respectively, in serovars Canicola, Pomona, Icteroahemorrhagiae, and Grypothyposa, in that order.

In chronic cases with the presence of antibodies against *Leptospira*, higher than one titer out of 100, the pattern of the lesion was characterized by interstitial nephritis; 57.89% (11/19) showed 100 titers; 36.84% (7/19) showed 200 titers, and 5.26% (1/19) 600 titers, in serovars Australis, Pomona, Canicola, Tarasovi, Pyrogenes, and Icteroahemorrhagiae, respectively. That lesion is a late finding of infection by leptospirosis in chronic carriers.

In seronegative or sick pigs due to causes other than leptospirosis, such as asymptomatic infections on noninfectious processes, the pattern of the lesion was characterized by glomerulitis, which can mean the beginning of other more intense renal alterations. These assertions are similar to the findings of Martínez *et al.* (2006), who noted that, besides *Leptospira* spp., there are other etiological agents that can cause interstitial nephritis, like the Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), Porcine Circovirus (PCV2), and Porcine Parvovirus (PPV), in addition to other bacteria such as *Streptococcus suis*, *Staphylococcus aureus*, and *Escherichia coli*, which intervene in purulent interstitial nephritis.

In this study, it was possible to corroborate the anatomopathological findings by means of serological diagnostic. Although silver staining (Warthin-Starry) is useful, there is little information available about the sensitivity and specificity of this technique (Azizi, Kheirandish, and Rahimi, 2014). Moreover, visualization of leptospirosis is more difficult due to a strong coloring on the bottom and the artifacts, which may produce false positives (Brihuega, 2010).

CONCLUSIONS

Glomerulonephritis, tubular nephritis, interstitial nephritis, and glomerulitis appear in the kidneys of asymptomatic pigs, positive or not to *Leptospira* spp, demonstrating the physiopathology, and broad spectrum of lesions caused by the infection in these animals.

The pigs with interstitial nephritis were 3.5-fold more likely to test positive by MAT than the ones that showed no lesion.

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Conception and design of research: M.P.Z.G, C.B.G, L.L.P, R.F.D, J.L.C.P; data analysis and interpretation: J M.P.Z.G, C.B.G, L.L.P, R.F.D, J.L.C.P; redaction of he manuscript: M.P.Z.G, C.B.G, L.L.P, R.F.D, J.L.C.P.

CONFLICT OF INTERESTS

The authors declare the existence of no conflicts of interests.