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Determination of *Leptospiral* antigens in naturally infected canine uterus by immunohistochemical immunofluorescence and ELISA methods

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Abstract

Leptospirosis is a zoonotic disease caused by various pathogenic *Leptospira* serovars. The disease also affects various animal species, especially humans. This disease, widespread in dogs, has become an important public health issue. In this study, uterine tissue and blood samples of 100 naturally infected dogs were examined to detect the presence of *Leptospiral* antigens. 100 uterine tissue samples were obtained from dogs that underwent ovariohysterectomy in Erzurum. After the uterine tissue was taken into buffered formalin solution, these samples were examined by histopathological, immunohistochemical, and immunofluorescence methods. The other part was putting it into the freezer at -20˚C for examinations made by ELISA (IgM and IgG). Blood samples were centrifuged and analyzed in the laboratory using the ELISA (IgG) method. In the histological examinations of uterine tissue samples to determine the cycle periods, findings of 57 proestrus, 14 estrus, 19 diestrus, and 10 anoestrus periods were determined. In the presented study, in uterine tissue samples, in the examinations performed with the immunohistochemical staining method 19%, in the examinations made with the immunofluorescence staining method 24%, and in the examinations performed with the ELISA diagnostic method IgM 4%, and IgG 23.52% were detected. When the blood samples were analyzed using by ELISA diagnostic method, 51% seropositivity was detected. In conclusion, Leptospirosis, found to be positive in both the blood and uterine tissues of dogs by different methods, was found to be quite common in dogs today, and it is thought that this zoonotic disease is a threat to public health.

Key Words: ELISA, Histology, Immunohistochemistry, Immunofluorescence, *Leptospira*

Introduction

Leptospirosis is one of the most important zoonotic diseases in the world because it causes crucial health problems in humans and animals, as well as economic losses in domestic animals[9]. Leptospirosis is a zoonotic disease caused by *Leptospira* serovars with various clinical symptoms in humans, pets, and rodents[6,14,32]. *Leptospires* in the *Leptospiraceae* family in the order of *Spirochaetales* are divided into two critical structures saprophytic *biflexia* and parasitic *interrogans*[20,47]. Within these two structures, there are antigenically distinct serotypes (serovars). The structures of serotypes carrying common antigens are classified as serogroups. While there are more than 300 serotypes and 26 serogroups showing pathogenic characteristics in *L. interrogans*

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species, there are more than 60 non-pathogenic serogroups in *L. bifleksia* species[21]. The Leptospira species causing the disease used to vary from region to region, but recently this variation has disappeared due to increased maritime transportation, natural disasters, and climate change. It is thought that the transportation of reservoir rodents to different regions has played a role in this picture. It has also been reported that the failure to provide important health protection measures such as sanitation, personal hygiene, health services, and vaccination has also played a role in the disappearance of differences[4]. In Turkey, as in other regions worldwide, it has been detected in numerous studies conducted on different species that the *L. interrogans* serovar and its associated serogroups are highly prevalent[4,7,37-39].

Transmission of the disease usually occurs in direct contact with the urine of sick animals or indirectly due to contact with contaminated materials[18,21]. Agents usually enter the body through skin portante or mucosal surfaces. Then, after an incubation period of 4-6 days, they mix with the blood and form leptospiremia[7]. After leptospiremia, disease agents may be localized in organs and tissues such as the kidney, liver, uterus, spleen, brain, testes, eye, and muscle[39,53]. High fever, chills, muscle weakness, vomiting, dehydration, tachypnea, and shock may be observed in this sickness. In addition, in some cases, depression, jaundice, hemoglobinuria, hemolytic anemia, and mucous membranes can be seen to cause pale and occasional bleeding[28,41]. It is known that *Leptospira* agents settle in genital organs and cause abortions in pregnant animals and clinical cases such as mastitis and orchitis[7,19,23,31]. However, the disease can sometimes show an asymptomatic course. Factors such as the host's age, species, and immune system, as well as the agent's type, amount, and virulence, can affect the emergence of clinical findings. For this reason, the disease can settle in many tissues and organs and remain in the living without showing any clinical picture. This bacteria can cause serious health problems as the disease spreads more quickly to the environment[9,10,33]. In the acute phase of the disease in dogs, abortion occurs in bitch. Abortions usually occur in the last stages of pregnancy, and in regions where the disease is common, the abortion rate can reach up to 20-30%. In abortion, the placenta cannot be expelled, and excessive thickening of the umbilical cord and membranes of the offspring is observed[1].

Macroscopic and microscopic findings are essential in the diagnosis of the disease. In the disease, jaundice and hemoglobinuria are frequently encountered with ecchymotic and petechial hemorrhages on the mucosal and serosal surfaces of organs and tissues macroscopically[55]. In the disease, histopathologically; Vasculitis and endothelial damage in vessels, inflammatory reactions consisting of mononuclear cells and neutrophil leukocytes in tissues are characteristic[37,38,46,53]. Apart from these diagnostic methods, bacteriological culture and agglutination tests are also widely used in diagnosing the disease[11,44]. In addition, molecular and immunohistochemical techniques have been widely used recently[20,38,39,46].

This study aims to reveal the *Leptospira* antigens in naturally infected canine uterine tissues for the first time by immunohistochemical, immunofluorescence, and ELISA methods.

Materials and Methods

Animals

The material of this study consisted of serum samples and uterine tissue samples obtained from 100 dogs that underwent ovariohysterectomy operation in Erzurum region. Erzurum is a region that receives significant rainfall during winter and spring. As these seasons come to an end, extensive swamp areas are formed in the region due to melting snow. The region has moderate temperatures and encompasses vast pasturelands. The population of stray dogs in the area is quite highly. It is known that the average age of these dogs is between 18-24 months and they have at least one estrus period. A blood sample and uterine tissue were taken from a suspected dog to be used in the study, and after the disease

positivity was determined by ELISA method, the uterine tissue was used as a positive control in the study. Blood sample and uterine tissue from a healthy dog were also used as negative control in the study after the disease was screened by ELISA method and found to be negative.

Macroscopic examinations

In the study, macroscopic examinations of uterine tissue samples were made, and samples were taken from the uterine horn region of the uterus for examination[13,42,51].

Histopathological examinations

Uterine tissues were fixed in 10% buffered formaldehyde solution for 48 hours. Tissues were followed routinely on a tracking device (Thermo Shandon Citadel 2000), and paraffin was blocked. Sections of 4 μm thickness were taken from the blocks to standard slides for histopathological examinations, and adhesive (polylysine) slides for immunohistochemical examinations with a microtome device (Leica RM2125 RT). All samples were stained with hematoxylin-eosin, and the tissues were examined under a light microscope (OLYMPUS BX51 JAPAN). In our study, in the histologic determination of the cycle;

In the proestrus period; bleeding in the mucosa, proliferation in the glands and structure of vessel

During estrus; proliferation of glands, desquamation of uterine mucosa epithelium and structure of vessel

During the diestrus period; decrease in glands and normal structure of mucosal epithelium and structure of vessel

During the anoestrus period; signs of a severe decrease in the number of glands and structure of vessel were taken into consideration.

Immunohistochemical staining method

Primary antibody (*Leptospira interrogans* U-Protein Express BV, Catalog No: P-Ab0006, Dilution Ratio: 1/100) was dripped to the uterine tissue samples to detect the presence of *Leptospira* antigen by immunohistochemical method, and the sections were kept in an oven at 37˚C for 1 hour. In the immunohistochemical method, 3-3' Diaminobenzidine (DAB) chromogen was used as the chromogen. After the sections were washed in distilled water, they were kept in Mayer's Hematoxylin solution for 1 minute for floor staining. Tissues were examined under a light microscope (OLYMPUS BX51 JAPAN), and pictures were taken of the sections with immunopositivity during the examination (OLYMPUS DP72 JAPAN).

Immunofluorescence staining method

In this method, the first primary antibody (*Leptospira interrogans* U-Protein Express BV, Catalog No: P-Ab0006, Dilution Ratio: 1/100) was dripped onto the sections, and the sections were kept in an oven at 37˚C for 1 hour. Then, the secondary immunofluorescence antibody FITC (Catalog No: ab6717, Dilution Ratio: 1/500) was dripped onto the sections and incubated in the dark for 45 minutes. The second primary antibody (LipL32 Catalog No: mbs505042, Dilution Ratio: 1/200) was dripped onto the sections and kept at 37˚C for 1 hour. Then, the secondary immunofluorescence antibody Texas Red (Catalog No: ab6787, Dilution Ratio: 1/500) was dripped onto the sections and kept in the dark for 45 minutes. DAPI (Catalog No: D1306, Dilution Ratio: 1/200) solution was dripped onto the sections and kept in the dark for 5 minutes. At the end of the staining, the sections were examined under a fluorescent microscope (ZEISS AXIO GERMANY), and the images of the immunopositivity areas were taken under a confocal microscope (Zeiss LSM 710 GERMANY). Immunopositivities were evaluated according to their severity as absent (-), mild (+), moderate (++), and severe (+++).

Enzyme-linked immuno sorbent assay (ELISA)

•Preparation of serum samples

Blood samples obtained from dogs with uterine tissues were centrifuged at 10,000 rpm for 10 minutes (BECKMAN COULTER Allegra X-30R). The obtained serum samples were taken into Eppendorf tubes, and the commercial kit procedure performed ELISA tests. *L. interrogans* IgG antibody (Canine *Leptospira* antibody (IgG) BT LAB ELISA Kit Cat no: ED0032Ca) was used

for these tests.

• Preparation of tissue samples

Homogenization of sections weighing 1 g from each canine uterus was performed in an automatic device (Roche MagNA Lyser) at 6,000 revolutions and 90 seconds. After homogenization, the tissues were centrifuged at 10,000 rpm for 10 minutes, and the supernatant solution was obtained. The ELISA test was performed on the supernatant solutions per the commercial kit procedure. For the detection of the presence of *Leptospira* antigen in uterine tissue samples by ELISA test method, *Leptospira interrogans* IgG antigen (Canine *Leptospira* antibody (IgG) BT LAB ELISA Kit Cat no: ED0032Ca) and *Leptospira interrogans* IgM antigen (Canine *Leptospira* antibody (IgM) BT LAB ELISA Kit Cat no: ED0033Ca) were used.

Preparing the ELISA test

First, 50 µl of the positive and negative control solutions in the kit were added to the wells. Then, 40 µl of standard solution and 10 µl of the prepared samples were added to the remaining wells. The prepared plate was incubated at 37˚C for 30 minutes. At the end of the incubation period, the plate was washed 5 times. After washing, 50 µl of conjugate was added to each well and incubated at 37˚C for 30 minutes, then the plate was washed. 50 µl of Solution A and Solution B in the kit were added to the wells, respectively, and incubated at 37˚C for 10 minutes. At the end of the incubation period, 50 µl of stop solution was added to each well without washing and incubated for 10 minutes in the dark. After these steps, the optical densities of the samples in each well were read in a microplate spectrophotometer (BioTEK EPOCH 2) with a 450 nm filter and the results were recorded.

Calculation of results;

Cutoff Value=avarage Negative Control value + 0.15

While ODsample < Cutoff Value: Negative

While ODsample ≥ Cutoff Value: Positive

Statistical analysis

Mann Whitney U test was used to compare binary groups of data obtained semiquantitatively. SPSS 20.0 program was used to determine these statistical analyzes. The chi-square test was performed to show the correlation between uterine cycle periods and *Leptospira* immunopositivity. In the chi-squared analysis, Proestrus and Estrus (V_1) periods were considered as one group and Diestrus and Anoestrus (V_2) periods as another group. ROC curve analysis was performed for the evaluation of ELISA serum findings.

Among the methods used in the study, the immunofluorescence method was used as the gold standard to evaluate the sensitivity and specificity. The reason for using the immunofluorescence method as the gold standard is that LipL32 protein expression increases the specificity in diagnosing pathological *Leptospira* agents in clinical samples. In the presented study, as a result of the detection of LipL32 with the immunofluorescence method, the immunofluorescence method was used as the gold standard in the comparison[24,29,30].

Results

Macroscopic findings

Macroscopic examination of canine uterine tissues revealed no specific lesions, but some tissues showed very mild signs of metritis. However, no correlation was observed between these mild lesions and the disease.

Microscopic findings

In the study, the histological findings observed in the uterine tissues were evaluated, and the cycle periods of the animals were determined. In the microscopic examination of the uterus samples, mild metritis was found in 11 samples.

Histological appearances of the examined uterus samples according to the cycle periods are followed as follows;

Proestrus period; It was observed that the uterine lumen was empty, and the epithelial tissue was normal. Hyperplasia and increased secretion were observed in uterine gland epithelial cells. Vasodilation and hyperemia were seen in the vessels (Figure 1).

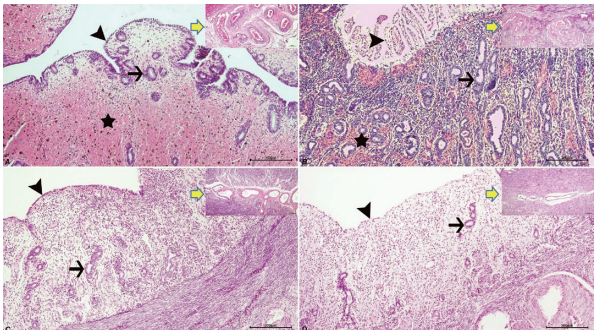

Fig. 1. Uterine tissue, Proestrus period (A), Oestrus period (B), Diestrus period (C), and Anestrus period (D). Uterine epithelial cell (arrowhead), uterine gland epithelial cell (arrow) and bleeding (star), changes in the vessels are shown in the upper right corner of the figures (yellow arrow), H&E, Bar: 200 µm.

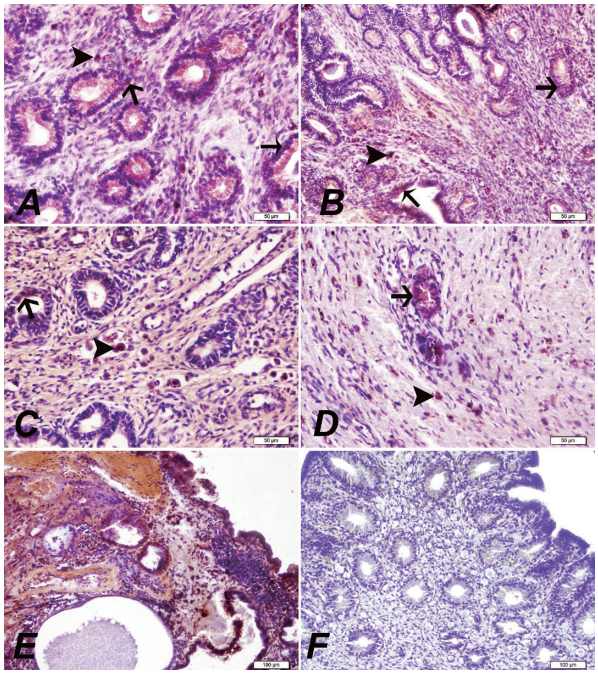

Fig. 2. Uterine tissue, Proestrus period (A), Oestrus period (B), Diestrus period (C), Anestrus period (D), Positive control (*Leptospira interrogans*) (E) and Negative control (F). In the cytoplasm of tissue macrophages (arrowhead), in the cytoplasm of uterine gland epithelial cells (arrow), and the cytoplasm of uterine epithelial cells (thin arrow), *Leptospira interrogans* immunopositivity IHC-P, Bar: 50 and 100 µm.

Estrus period; The typical finding was the presence of large shed epithelial cells in the uterine lumen. It was observed that the density of these epithelial cells decreased in the following periods. It was also observed that the secretory glands were active in the early phase of this period. Vasodilation and mild hyperemia were observed in the vessels (Figure 1).

Diestrus period; In this period, it was observed that the uterine lumen was empty, and the epithelial cells were in a standard structure. It was noted that secretion had decreased with atrophy in the glands, and the signs of hyperemia had disappeared with vasoconstriction in the veins (Figure 1).

Anoestrus period; In this period, it was determined that the uterine lumen was empty and the epithelial cells were in a typical structure, a decrease in the number of glandular structures was formed, and accordingly, there was no secretion. Blood vessels were selected in standard vascular structure (Figure 1).

As a result of histological examinations in uterine tissues of 100 samples, It was determined that there were 57 proestrus, 14 estrus, 19 diestrus, and 10 anoestrus periods.

Immunohistochemical findings

Immunopositivity was detected in 19 (19%) of 100 uterine tissue samples examined by the immunoperoxidase staining method. This immunopositivity; was found in the cytoplasm of uterine mucosal epithelial cells, uterine gland epithelial cells, and macrophages observed in the endometrium layer (Figure 2). Of these immunopositive, 11 were found in proestrus, 4 in estrus, 3 in diestrus, and 1 in anoestrus. As a result of the statistical analysis, there was no significant difference between uterine cycle periods and IHC immunopositivity (($P = 0.575$) $P > 0.05$) (Expected value: V_1: Negative 78.9%, 21.1 Positive % and Total 100.0%; V_2: Negative 86.2%, 13.8 Positive % and Total 100.0%; Total: Negative 81.0%, 19.0 Positive % and Total 100.0%). In the study, the sensitivity and specificity of immunohistochemical analyzes were 41.66% and 88.15%.

Immunofluorescence findings

Immunopositivity was detected in 24 (24%) of 100 uterine tissue samples examined by the immunofluorescence staining method. By immunopositivity, It was found in the cytoplasm

of uterine mucosal epithelial cells, uterine gland epithelial cells, and macrophages observed in the endometrium layer. LipL32 membrane protein expression was investigated by the immunofluorescence method in uterine tissue samples to confirm the microscopic examinations. LipL32 membrane protein was detected in 24 uterine tissues positive for *Leptospira* in the immunofluorescent staining method (Figure 3). In scoring, There was no statistically significant difference between *Leptospira* agent immunopositivity and LipL32 protein expression ($P > 0.05$). It was determined that 12 of 24 samples with *Leptospira* immunopositivity were in proestrus, five were in oestrus, five were in oestrus, and two were in anoestrus. As a result of statistical analysis, no significant difference was found between the uterine oestrus cycle and immunopositivity (($P = 1.00$) $P > 0.05$) (Expected value: V_1: Negative 76.1%, 23.9 Positive % and Total 100.0%; V_2: Negative 75.9%, 24.1 Positive % and Total 100.0%; Total: Negative 76.0%, 24.0 Positive % and Total 100.0%).

ELISA review findings

In 51 (51%) of the serum samples examined, seropositivity was detected to determine the IgG antibody formed against *Leptospira* agents. As a result of the statistical analysis, the cut-off point was found to be > 0.216, and it was observed that the positive values obtained were greater than this value (Table 1). A positivity was detected in 4 (4%) of the uterine samples examined to detect IgM antibodies in uterine tissues. When the uterus samples of 51 samples with seropositivity in IgG antibody screening were examined by ELISA (IgG) test method, positivity was detected in 12 (23.52%) of the uterus samples. In the uterine tissue, the sensitivity of IgG was 8.33%, and the specificity was 88.15%. The examination results of Histopathological, Immunohistochemical, Immunofluorescence and ELISA methods using blood and uterus samples are presented in Table 2.

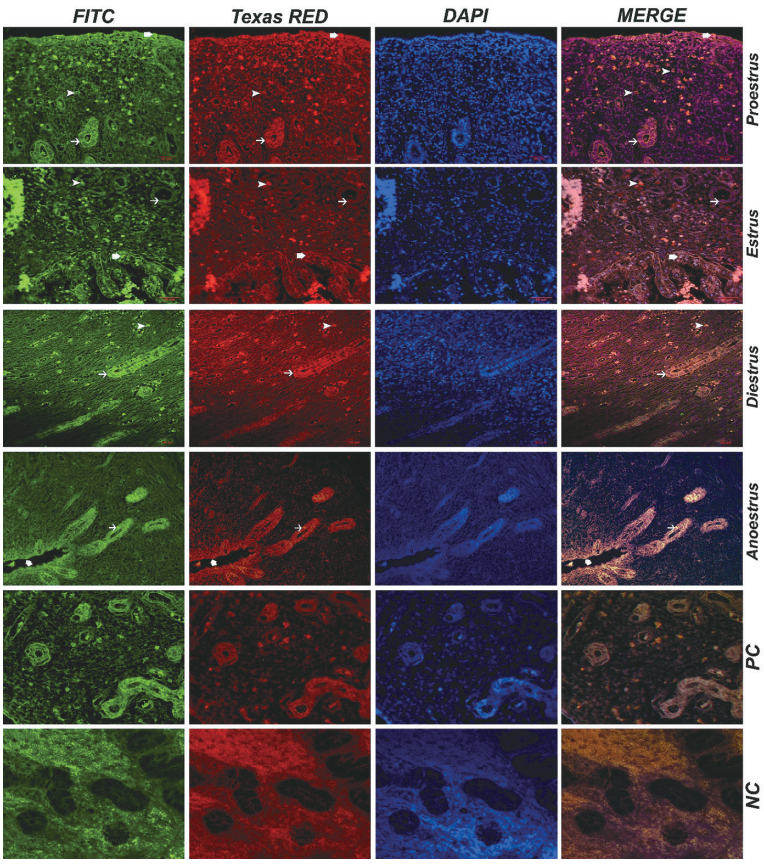

Fig. 3. Uterine tissue; *Leptospira interrogans* immunopositivity (FITC) in the cytoplasm of tissue macrophages (arrowhead), in the cytoplasm of uterine gland epithelial cells (thin arrow), in the cytoplasm of uterine epithelial cells (thick arrow). LipL32 protein expressions (Texas RED) in the cytoplasm of tissue macrophages (arrowhead), in the cytoplasm of uterine gland epithelial cells (thin arrow), in the cytoplasm of uterine epithelial cells (thick arrow), PC: positive control (FITC: *Leptospira interrogans;* Texas Red: LipL32), NC: negative control, IF, Bar: 50 µm.

Table 1. The cut-off point of the samples that were positive in serum samples by ELISA method

IgG Blood ELISA (*Leptospira interrogans*)	Value
AUC	1,000
Cut off	> 0.216
Specificity	98.04
Sensitivity	100.00
Significance level *P* (Area = 0.5)	< 0.0001

n = 100, 51 positive, 49 negative samples

Table 2. General results of histopathological, immunohistochemical, immunofluorescence, and ELISA (Blood and Tissue) examinations in uterine tissue samples

PN	Cycle Period	IHC	IF	ELISA Blood (IgG)	ELISA Uterus (IgG)	ELISA Uterus (IgM)
1	Estrus	+	+	-	-	-
2	Diestrus	-	-	-	-	-
3	Anoestrus	-	-	+	-	-
4	Diestrus	-	+	+	-	-
5	Diestrus	-	-	+	-	-
6	Diestrus	-	+	+	-	+
7	Diestrus	-	-	-	-	-
8	Diestrus	-	-	-	-	-
9	Diestrus	-	-	+	-	-
10	Anoestrus	+	+	+	+	-
11	Anoestrus	-	-	+	+	-
12	Proestrus	-	-	+	-	-
13	Diestrus	+	-	-	-	-
14	Anoestrus	-	-	+	-	-
15	Diestrus	-	-	-	-	-
16	Diestrus	+	+	+	+	-
17	Proestrus	-	-	-	-	-
18	Proestrus	-	-	-	-	-
19	Proestrus	-	-	-	-	-
20	Diestrus	-	-	+	-	-
21	Proestrus	-	+	+	-	-
22	Diestrus	+	-	-	-	-
23	Estrus	-	-	+	-	-
24	Diestrus	-	-	+	-	-
25	Diestrus	-	-	-	-	-
26	Diestrus	-	+	-	-	-
27	Diestrus	-	+	-	-	-
28	Estrus	-	-	-	-	-
29	Anoestrus	-	+	+	-	-
30	Anoestrus	-	-	-	-	-
31	Diestrus	-	-	-	-	-
32	Diestrus	-	-	+	-	-
33	Diestrus	-	-	-	-	-
34	Proestrus	+	+	-	-	-
35	Proestrus	-	-	-	-	-
36	Proestrus	-	-	+	-	-
37	Estrus	-	-	-	-	-
38	Anoestrus	-	-	+	-	-
39	Proestrus	-	+	-	-	-
40	Proestrus	-	-	-	-	-
41	Estrus	-	-	-	-	-
42	Proestrus	-	-	-	-	-
43	Proestrus	-	-	+	-	-
44	Proestrus	+	+	+	+	-

PN	Cycle Period	IHC	IF	ELISA Blood (IgG)	ELISA Uterus (IgG)	ELISA Uterus (IgM)
45	Proestrus	-	+	-	-	-
46	Proestrus	-	-	+	-	-
47	Proestrus	-	-	+	-	-
48	Estrus	+	-	-	-	-
49	Anoestrus	-	-	-	-	-
50	Proestrus	-	+	-	-	-
51	Proestrus	-	-	+	-	-
52	Proestrus	-	-	-	-	-
53	Estrus	-	-	-	-	-
54	Proestrus	-	-	+	-	-
55	Proestrus	-	+	+	-	-
56	Proestrus	-	-	-	-	-
57	Estrus	-	+	+	-	-
58	Proestrus	+	+	+	+	-
59	Estrus	-	+	-	-	-
60	Proestrus	-	-	+	-	-
61	Proestrus	-	-	-	-	-
62	Proestrus	-	-	-	-	-
63	Proestrus	-	-	+	-	-
64	Proestrus	+	+	+	+	-
65	Proestrus	-	-	-	-	-
66	Estrus	+	-	-	-	-
67	Proestrus	-	-	-	-	-
68	Proestrus	+	-	+	-	-
69	Estrus	-	-	+	-	-
70	Anoestrus	-	-	-	-	-
71	Proestrus	-	-	-	-	-
72	Proestrus	-	-	-	-	-
73	Proestrus	-	-	+	+	-
74	Proestrus	-	-	-	-	-
75	Proestrus	-	-	+	-	-
76	Estrus	-	+	+	-	-
77	Proestrus	-	-	+	-	-
78	Proestrus	+	+	+	+	-
79	Estrus	-	+	-	+	-
80	Proestrus	-	-	-	-	-
81	Proestrus	-	-	+	-	-
82	Proestrus	-	-	+	-	-
83	Proestrus	-	-	-	-	-
84	Proestrus	-	-	+	-	-
85	Proestrus	+	+	+	+	-
86	Proestrus	+	-	+	-	-
87	Proestrus	-	-	+	-	-
88	Proestrus	-	-	+	-	-
89	Proestrus	-	-	+	-	-
90	Anoestrus	-	-	-	-	-
91	Proestrus	-	-	+	-	-
92	Proestrus	-	-	+	-	-
93	Proestrus	-	-	-	-	-
94	Proestrus	+	-	-	-	-
95	Proestrus	+	+	+	+	-
96	Proestrus	-	-	+	-	+
97	Proestrus	-	-	-	-	-
98	Proestrus	-	-	+	+	-
99	Proestrus	+	-	+	-	+
100	Estrus	+	-	-	-	+

Discussion

Leptospirosis is a zoonotic disease caused by *Leptospira interrogans* serovars[11,15,16]. This disease is seen in many animals, such as cattle, sheep, horses, pigs, cats, dogs, and mice, especially humans[27,37,38,53]. In the seroprevalence studies conducted in different parts of the world on the presence and prevalence of the disease, leptospirosis disease; was found at a rate of 21.1%[43] in Poland, 18.1%[8] in Romania and 22.3%[22] in Bosnia and Herzegovina. In Turkey, seropositivity was reported as 11%[48] in Bursa, 7%[4] in Istanbul, and 33%[50] in another study covering the country. In this study, the seroprevalence of the disease in dogs was found to be 51%, and this rate was higher than previously reported. In line with the data obtained from the studies, it has been observed that leptospirosis disease is widespread worldwide and varies at high rates between regions. In the presented study, the ELISA IgG cut-off point in blood samples was > 0.216, and the values found to be positive in the study were higher than this value. Again, in the literature review, no study such an examination was found in dogs, and new information was brought to the literature with this study. It is thought that the stray dogs used in the study were not vaccinated against the disease agent. Despite this, it is thought that the antibody rate in animal serums is so high because of the animals' natural immunity against the disease.

As can be seen in the reports reported above[4,8,22,43,48], the ELISA method used in the diagnosis of *Leptospira* disease in dogs has mostly been studied on body fluids[5,17,32]. However, no study was found in which *Leptospira* agents were investigated by the ELISA method in canine uterus tissue. In this presented study, canine uterus tissues were examined for the first time for leptospirosis by ELISA (IgM and IgG) method, and the presence of 4% IgM and 23.52% IgG antigen was detected.

The histopathological changes caused by leptospirosis in the uterus have been demonstrated by a limited number of studies in different animal species[25,51,56]. In these studies, histopathologically intense perivascular and periglandular lymphocytic cell infiltrates in the *Leptospira* infected uterine tissue, widespread edema and congestion in the tissue, as well as intense hydropic degeneration of the uterine gland epithelium have been reported[25,51,56]. This study was conducted on naturally infected canine uterine tissues; Although neutrophil leukocyte cell infiltrates were similar to the histopathological findings reported in other studies, their severity was not as high as reported values. In light of this result, it was thought that *Leptospira* agents could settle in the uterine tissue with an asymptomatic course.

Leptospira disease has been reported to result in clinically severe problems and death in dogs. The literature review shows that the disease causes severe symptoms such as icterus, anemia, and hemoglobinuria in dogs[26,49]. However, in some cases, it is known that the disease has a clinically asymptomatic course[10,33]. In this study, the disease agent was detected in the uterine tissues that were not damaged in both macroscopic and microscopic examinations. In light of the literature and the findings obtained from the study, it has been observed that the disease can show a clinically asymptomatic course in dogs. Again, these results suggest that taking protective measures against this zoonotic disease will be necessary for public and animal health. It has been reported that when leptospirosis disease is asymptomatic in canine kidney tissues, it may cause the disease to spread to the environment via urine and infect intermediate hosts such as humans[35]. Depending on the asymptomatic course of the positive uterine tissues in this study, it is thought that the spread of the disease to the environment may be easy with body secretions such as abortion and uterine discharge, and accordingly, the disease may be transmitted to intermediate hosts such as humans.

The studies focused on Leptospirosis disease using immunohistochemical methods; The immunopositivity rates were 35%[3] in cattle kidneys, 33.33% in bovine aborted fetus kidneys, and 23.07% in livers of these fetuses[37]. In studies conducted with the IHC staining method on

leptospirosis disease in dogs, In 18.1%[36] of the kidney samples, in the lung tissues 11.5%[41] and in the testicular tissues 26.66%[7] immunopositivity was detected. In this study, the uterine tissues of the dog were examined by the IHC staining method, and 19% immunopositivity was found. The fact that these values in the study are close to those reported in previous studies shows that the disease is still at high rates today.

Although the immunofluorescence staining method is not widely used in the diagnosis of leptospirosis disease, there are very few studies[5,39]. With the immunofluorescence method, the rate of immunopositivity was 43.96%[5] on blood serums in dogs and 7.14%[39] on cattle kidneys. In this study, 24% immunopositivity was detected in canine uterus tissues by the immunofluorescence staining method. In this study on canine uterine tissues, although it was observed that most of the positive samples in IHC and IF methods were in the proestrus and estrus periods, no statistically significant difference was found. These results are thought to be due to the large number of animals used in the study and the variable number of cycles determined. In addition, this is the first study in which canine uterus tissue samples were examined for leptospirosis by immunohistochemical methods (IP, IF). In many studies investigating *Leptospira* infections from tissues[45,52] and body fluids[12,34,40], it has been reported that the presence of LipL32 membrane protein is vital for the diagnosis of the disease. In this study, LipL32 protein expression in all 24 *Leptospira* positive samples by immunofluorescence method reveals that detecting LipL32 protein is vital in diagnosing the disease.

The MAT test is the gold standard in diagnosing *Leptospira* infections[2,54]. However, in recent studies, it has been reported that IHC and IF tests are also effective in diagnosing the disease in terms of sensitivity and specificity[39]. In addition, LipL32 protein expression was found to increase specificity in diagnosing pathological *Leptospira* agents in clinical samples. In this study, LipL32 protein expressions were revealed with the IF method, and for this reason, the IF method was accepted as the gold standard in the study[24,29,30]. Accordingly, it has been demonstrated that the IHC method is more sensitive than the ELISA method in detecting disease agents in uterine tissue. This situation is thought to be caused by the intracellular localization of *Leptospira* agents.

As a result, with this study, leptospirosis disease was determined for the first time in canine uterus tissues by IF, IHC, and ELISA methods, and new information was provided to the literature. At the end of the study, it was seen that the ELISA method made from serum samples in the chronic period is more sensitive in diagnosing the disease. The immunofluorescence staining method was more sensitive than other methods in detecting disease agents in uterine tissues. As a result of the study, it is thought that the disease may cause significant problems in terms of public health, considering that the disease is quite common in dogs and the ritual of growing dogs at home has become widespread recently.

Declarations

Ethical approval

The work permit was approved by the Atatürk University Rectorate Faculty of Veterinary Ethics Committee (Number of Ethics: 2019/14).

Consent to participate

Not applicable.

Consent for publication

All authors had approved the final version of the manuscript for publication.

Availability of data and materials

All of the data used in the study can be accessed from the article content.

Competing interests

The authors declared no potential competing interest with respect to the research, authorship, and/or publication of this article.

Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' contributions

- **Ismail Bolat:** Collection and preparation of tissues, staining and evaluation of tissues by immunohistochemical methods, writing of the article.
- **Yavuz Selim Saglam:** Staining and evaluation of tissues by immunohistochemical methods, writing of the article.
- **Seyda Cengiz:** Application and evaluation of ELISA method in tissues.
- **Serkan Yildirim:** Collecting and preparing arrows, staining and evaluation of tissues by immunohistochemical methods.

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