ISSN: 1220-3173; E-ISSN: 2457-7618

THE HEALTH STATUS OF DOGS REGARDING THE INFECTION WITH *LEPTOSPIRA* SPP.

STAREA DE SĂNĂTATE A CÂINILOR INFECȚAȚI CU *LEPTOSPIRA* SPP.

C. ȘONEA[1)], Maria Rodica GURĂU[1),*)], I.B. HOBU[1)], F. OȚELEA[2)], J. YAZBAK[1)], Raluca-Aniela GHEORGHE-IRIMIA[1)], Georgeta ȘTEFAN[1)]

ABSTRACT | REZUMAT

Leptospirosis is a zoonosis prevalent all over the world; caused by a spirochete belonging to the genus *Leptospira*, it affects a wide range of animal species. This work presents the results of a study regarding *Lepotspira* infection in dogs within the Bucharest and Ilfov county area. The detection of dogs with *Leptospira* was carried out following the Classical PCR test results. In the group of dogs studied, 18.18% of the samples tested positive for the presence of the specific DNA fragment of *Leptospira* spp. On the other hand, 81.82% of the samples tested negative, indicating a low prevalence of leptospirosis within the studied dog population.

Keywords: Canine leptospirosis, PCR, diagnosis

Leptospiroza este o zoonoză răspândită în întreaga lume, cauzată de o spirochetă aparținând genului *Leptospira*, care poate afecta numeroase specii de animale. Această lucrare prezintă rezultatele unui studiu privind infecția cu *Leptospira* la câini din raza municipiului București și județului Ilfov. Detectarea câinilor infectați cu *Leptospira* a fost realizată prin PCR clasic. În lotul de câini studiat, 18,18% dintre probe au fost testate pozitiv pentru prezența fragmentului de ADN specific de *Leptospira* spp. Pe de altă parte, 81,82% dintre probe au fost testate negative, indicând o prevalență scăzută a leptospirozei în cadrul populației de câini studiate.

Cuvinte cheie: leptospiroza canină, diagnostic, PCR

Leptospirosis is a zoonosis found all over the globe. Produced by a spirochete belonging to the genus *Leptospira*, it affects a wide range of animal species. The epidemiology of leptospirosis has been influenced by various factors, including alterations in animal husbandry practices, shifts in climate patterns, and changes in human behaviour. Due to the outbreaks of leptospirosis in the last decade, the disease has enjoyed increased interest recently, which led to the revision of the complex taxonomy of *Leptospira*, previously classified by serology and recently modified by a genotypic classification (4). Also, the importance of early antibiotic therapy has led to the development of several simple and rapid diagnostic tests. The transmission of *Leptospira* takes place when infected animals release the bacteria through their urine, thereby contaminating the surrounding environment (4).

Leptospires are motile helical bacteria, ranging in size from 0.1 x 6 to 12 pm, and feature hook-shaped ends. While they are cytochemically Gram-negative, they do not stain effectively with conventional bacteriological dyes. As a result, visualising *Leptospira* typically requires the use of dark-field microscopy (2).

Leptospires are corkscrew-shaped bacteria, distinct from other spirochaetes by the presence of end hooks. They belong to the order of *Spirochaetales,* family *Leptospiraceae,* genus *Leptospira*. Leptospires typically have a diameter of around 0.1 µm and vary in length from 6 to 20 µm (5).

The serovars associated with leptospirosis in dogs are *L.* Canicola and *L.* Icterohaemorrhagiae (1, 8). However, due to the extensive utilisation of vaccines containing these serovars, the emergence of the serovars *L.* Grippotyphosa and *L.* Pomona has become significant as canine pathogens. Serovar *Canicola*, specifically adapted to infect dogs, leads to severe renal disease in puppies. In surviving animals, there is a possibility of developing a chronic uremic syndrome following the acute phase (8, 10). On the other hand, incidental infections in dogs, typically caused by serovar *L.* Icterohaemorrhagiae, manifest as either acute hemorrhagic disease or subacute hepatic and renal failure. In canine infections caused by serovars other than *L.* Icterohaemorrhagiae or *L.* Copenhagenii, renal symptoms are typically more prominent (1, 8).

Serovar *L.* Bratislava, specifically linked to abortion and infertility, is believed to be adapting to dogs, potentially acting as maintenance hosts. Vaccines containing only the serovars *L.* Icterohaemorrhagiae and *L.* Canicola do not confer immunity against other serovars (8).

1) University of Agronomic Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Bucharest, Romania
2) College of Romanian Veterinarians, Brăila County Branch, Romania
*) *Corresponding authors: otelea_maria@yahoo.com*

Leptospira can enter the host's body through openings in the skin or mucous membranes (Ellis, 1995). Carrier rats and mice are recognised as significant reservoirs of the disease. Leptospirosis in humans can manifest itself either as a flu-like, usually mild form of the disease, or as a severe, acute form with liver, kidney, and heart failure, which can lead to death. Leptospirosis has an economic impact on society due to farm animal costs and public health, with abortions, reduced production, and death occurring. Available vaccines have a low efficiency and duration; therefore, prevention through strict biosecurity measures is very important, the major objective being the control of rodents, considered the reservoir of this disease (10).

The diagnosis of bacterial diseases through molecular biology techniques has experienced a remarkable evolution in Romania, they are found in diagnostic protocols that use the most varied biological samples, from vector ticks to blood and urine samples (1, 6, 7, 9). Also, previous studies have demonstrated the usefulness of using urine samples in the detection of *Leptospira* spp by PCR (1).

The purpose of this work was to establish the status of *Leptospira* infection in dogs by detecting the bacteria in urine samples using molecular biology methods and to establish a link between the results obtained by PCR and the clinical signs recorded in dogs.

MATERIALS AND METHODS

Animal and biological samples

Table 1

Characteristics of dogs from which biological samples were taken for PCR analyses

#	Age	Sex	Breed	Location
1	3 years	F	Akita Inu	Bucharest
2	6 months	F	Akita Inu	Bucharest
3	10 months	M	Bulldog	Bucharest
4	4 years	M	Husky	Bucharest
5	4.5 years	F	Malinois	Bucharest
6	3.5 years	F	French Bulldog	Bucharest
7	1.5 years	M	Mix breed	Bucharest
8	7.5 years	M	Shih Tzu	Ilfov
9	5.5 years	M	Bichon	Ilfov
10	6 years	F	Mix breed	Ilfov
11	1 year	F	Shiba	Bucharest

Urine samples were collected for analysis from 11 dogs within the Bucharest and Ilfov county areas. The samples were collected in urine jars using disposable gloves for each individual animal subject. Samples were stored at 2-4°C until primary processing. Dogs were of various breeds. Six dogs were females and five were males. Three dogs were under the age of 1 year; five of the dogs were between 1 and 5 years of age; and three dogs were older than 5 years (Table 1).

Every urine sample was analysed using the same flow of work for DNA extraction and DNA amplification, and in order to be able to perform the PCR reaction for the detection of the specific fragment of leptospiral DNA, the hybridization temperature of the primers was previously optimised.

DNA extraction

To carry out the DNA isolation, the use of standardised kits offered the possibility of fast and accurate isolation. For the present research, the manual method was used using the commercial kit: PureLink® Genomic DNA Kits (3). The DNA extraction step was performed in a separate room dedicated to this procedure, in a laminar flow hood.

DNA amplification

The reagents and equipment:

- dNTP (200 µM);
- $MgCl_2$ (25 mM);
- 10X PCR buffer;
- Taq platinum polymerase (5 U/µL);
- Ultrapure water;
- Sense and antisense primers (10 pmol);
- Thermocycler IQCycler;
- MiniSpin microcentrifuge;
- REAX Top shaker.

The reaction mix was prepared for the desired number of tests (the number of samples plus one). The sequences of the primers used and the locus of the fragment in the genome for the sense primer is from codon 38 to codon 57 and for the antisense primer from codon 348 to codon 369 (Table 2).

Table 2

Primer's sequence used for amplification of *Leptospira sp.* DNA sequence (1, 6, 7)

Name	Primer sequence	Position in the genome
A	SENSE 5′GGCGCGCGTCTTAAACATG-3′	38 to 57
B	ANTI-SENSE 5′TTCCCCCCATTGAGCAAGATT-3′	348 to 369

Reagents were mixed in a 0.5 ml PCR tube, vortexed, and centrifuged according to the working protocol (Table 3).

Electrophoresis

The reagents and equipment:

- electrophoresis buffer: TAE 1X – TRIS 0.040 M, acetic acid 0.040 M, EDTA 0.002 M. It is prepared as a 10X solution, which is then diluted;

- sample buffer: bromophenol blue 0.3%, glycerol 30% in TAE 1X;
- ethidium bromide solution;
- molecular mass marker;
- agarose;
- horizontal electrophoresis tank for nucleic acids;
- power source;
- UV reading system (6).

Table 3

The working protocol for amplifications

The reaction/sample mix		DNA 50ng/ reaction/ sample	Spin
Water (RNase free)	17.75 µL	2 µl	**3000rpm /15 seconds**
PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.0)	2.5 µL		
$MgCl_2$ (1.5 mM)	0.75 µL		
dNTP solution			
(200 µM) (Invitrogen®, Carlsbad, California, USA)	0.5 µL		
Primer A (10 pmol)	0.5 µL		
Primer B (10 pmol)	0.5 µL		
Water (RNase free)	17.75 µL		

* Total volume per reaction/sample 25 µl

RESULTS AND DISCUSSIONS

As a result of the analysis carried out on the samples, the presence of leptospirosis infection could be outlined as a positive or negative PCR result (Table 4), and certain aspects may be discussed regarding the findings.

Table 4

PCR results and clinical status of dogs included in the study

Nr.	Symptoms	PCR Result
1	Asymptomatic	Negative
2	Asymptomatic	Positive
3	Asymptomatic	Negative
4	Asymptomatic	Negative
5	Diarrhoea	Negative
6	Asymptomatic	Positive
7	Asymptomatic	Negative
8	Asymptomatic	Negative
9	Asymptomatic	Negative
10	Asymptomatic	Negative
11	Asymptomatic	Negative

The two samples that are highlighted as positive results within the entire eleven samples batch (Fig. 1 and Fig. 2) are very important while discussing the presence of leptospirosis infection in the Bucharest area, while asymptomatic overall.

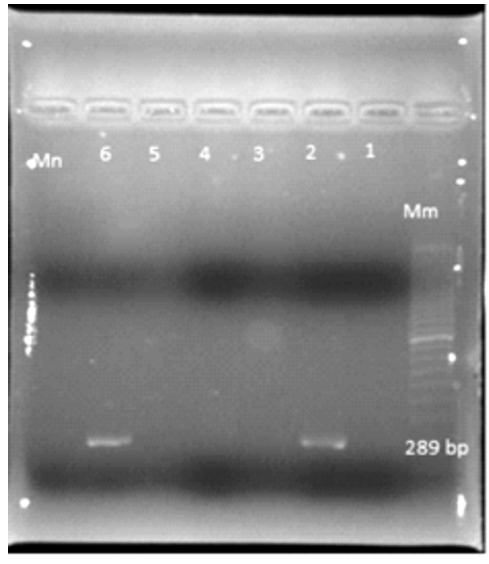

Fig. 1. The PCR results of the six-urine sample tested. The image is an electrophoresis results by visualising with UV. Mm= molecular marker by 100 bp, 1= sample 1, 2= sample 2, 3 = sample 3, 4 = sample 4, 5 = sample 5, 6= sample 6, Mn= negative control. Samples 2 and 6 are low positive, because it is shown a white line in front of the 289 bp

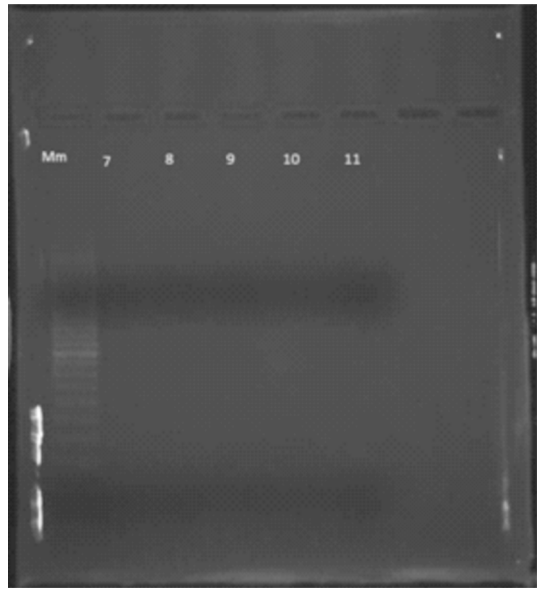

Fig. 2. The PCR results of the five-urine sample tested. The image is an electrophoresis results by visualizing with UV. Mm= molecular marker by 100 bp, 7= sample 7, 8= sample 8, 9 = sample 9, 10 = sample 10, 11= samples 11. There is no white line meaning all the samples are negative for the detection of leptospirosis

While breaking down the data into particular elements of interest, it is obvious that the male subjects

in the study group might be less prone to leptospirosis infection than the females. The proportion of male to female cases is lower. Furthermore, the proportion of female positive results is higher than that of male dogs. When the data is examined regarding the age of the subjects in the study group, it seems that the younger dogs are more likely to develop a leptospirosis infection than the elderly ones.

In this study, 50% positive results were recorded amongst young individuals, and 11.1% were recorded amongst adult dogs above the age of 1 year old.

In a similar study, 40.25% (31/77) samples were PCR-positive for *Leptospira* spp (1), but the targeted dogs were from 4 different shelters that collect free-ranging urban dogs from south-western Romania.

CONCLUSIONS

In the group of dogs studied, 18.18% of the samples tested positive for the presence of the specific DNA fragment of *Leptospira spp.* On the other hand, 81.82% of the samples tested negative, suggesting a low prevalence of *Leptospira* in the studied areas. Moreover, all positive dogs had no clinical signs of the disease at the time of collecting the urine samples.

REFERENCES

1. *Baraitareanu S., Gurau M.R., Danes D.,* (2014), Survey of *Leptospira* spp. in captive street dogs housed in shelters. Advances in Environmental Technology and Biotechnology. Energy, Environmental and Structural Engineering, 26:193-197
2. *Brenner D.J., Kaufmann A.F., Sulzer K.R., Steigenvalt A.G., Rogers F.C., Weyant R.S.*, (1999), Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for Leptospira alexanderi sp. nov. and four new *Leptospira* genomospecies. International journal of systematic bacteriology, 49(Pt 2):839-858
3. *Chevohn J.* (2012), PureLink® Genomic DNA Kits, Life Technologies Corporation, Available at: https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fpurelink_genomic_man.pdf (Accessed: 12.112.2021)
4. *Ellis W.A.,* (1995), International Committee on Systematic Bacteriology. Subcommittee on the Taxonomy of *Leptospira*. Minutes of the Meetings, 1 and 2 July 1994, Prague, Czech Republic. International Journal of Syslematic Bacteriology, 45:872-874
5. *Faine S., Adler B., Bolin C., Perolat P.,* (1999), *Leptospira* and leptospirosis,(Ed.)MediSci, Melbourne, Australia, 259
6. *Gurau M.R., Dragan M.S.,* (2018), Detection of the *Leptospira* genome in a Romanian pig farm. Revista Romana de Medicina Veterinara, 28(3):32-35
7. *Merien F., Amouriaux P., Perolat P., Baranton G., Saint Girons I.*, (1992), Polymerase chain reaction for detection of *Leptospira spp*. in clinical samples. Journal of Clinical Microbiology, 30(9):2219-2224
8. *Rentko V.T., Clark N., Ross L.A., Schelling S.H.,* (1992), Canine leptospirosis: a retrospective study of 17 cases. Journal of Veterinary Internal Medicine, 6(4):235-244
9. *Vladimirescu A., Dumitrescu G., Ionescu L., Necsulescu M., Moraru V., Popescu D., Bicheru S., Danes D., Baraitareanu S., Ciulacu-Purcarea V., Nicolescu G.,* (2016), Real-Time PCR studies regarding the *Borrelia burgdorferi*, *Francisella tularensis*, Tick borne encephalitis virus (TBEv) and Crimeean Congo hemorrhagic fever virus (CCHFv) occurrence in the Romanian ticks. International Journal of Infectious Diseases, 45(Supplement 1):193-194
10. *Yadeta W., Bashahun M., Abdela N.,* (2016), Leptospirosis in animal and its public health implications: a review. World Applied Sciences Journal, 34:845-853.