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# RESEARCH ON THE LEPTOSPIRA INFECTION PREVALENCE IN STRAY DOGS IN A ROMANIAN SHELTER

## CERCETĂRI PRIVIND PREVALENȚA INFECȚIEI LEPTOSPIRICE LA CÂINI COMUNTARI DINTR-UN ADĂPOST DIN ROMÂNIA

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**ABSTRACT**

Leptospirosis is a zoonotic disease with a wide range of clinical manifestations, from mild subclinical infection to multiple organ failure and death. Studies have shown that many dogs can be asymptomatic carriers, and they are involved in the spreading of *Leptospira* spp. in the environment through the infected urine. The aim of this study was to assess the usefulness of active surveillance of *Leptospira* spp infection in stray dogs, from the community shelters, by PCR analysis of non-invasively collected urine samples, by using cotton pads. The specific DNA-sequence of *Leptospira* spp. was detected by PCR assays in 86% of the urine samples. The microscopic agglutination test (MAT) has shown a 94.74% seroprevalence of *Leptospira* infection with 68.42% of dogs showing at least two *Leptospira* serovars inventions. Three different serovars were identified in the shelter: *L.* Bataviae (94.74%), *L.* Ballum (68.42%) and *L.* Australis (5.26%). PCR assay and the urine-cotton pad sampling method can be effective tools for controlling and preventing infection in humans and other animals by periodically testing stray dogs in the community shelters.

**Keywords**: leptospirosis, *Leptospira*, dogs, urine, diagnostic

**REZUMAT**

Leptospiroza este zoonoză care se manifestă clinic extrem de variat, de la infecții subclinice ușoare până la insuficiență severă și deces. Studiile au arătat că mulți câini pot fi purtători asimptomatici și sunt implicați în răspândirea *Leptospira* spp. în mediul înconjurător prin urina infectată. Scopul acestui studiu a fost evaluarea utilității supravegherii active a infecției cu *Leptospira* spp la câinii fără stăpân, din adăposturi comunitare, prin analiza PCR a probelor de urină colectate neinvaziv, folosind discuri de bumbac. Cu ajutorul testelor PCR, secvența ADN specifică *Leptospira* spp. a fost detectată în 86% probe de urină. Seroprevalența detectată prin testul de aglutinare microscopică (MAT) a fost de 94,74%. Rezultate serologice pozitive la cel puțin două serovaruri diferite au fost înregistrate la 68,42% din probele de ser cercetate. Au fost identificate trei serovari: *L.* Bataviae (94,74%), *L.* Ballum (68,42%) și *L.* Australis (5,26%). Analiza PCR și metoda de recoltare neinvazivă a probelor de urină cu ajutorul discurilor de bumbac pot fi utilizate in programele de control și prevenire a infecțiilor la om și alte animale prin testarea periodică a câinilor fără stăpân din adăposturile comunitare.

**Cuvinte cheie**: leptospiroză, *Leptospira*, câini, urină, diagnostic

Leptospirosis is a worldwide zoonosis caused by the infection with the pathogenic *Leptospira* species (stereotypes) (9). Several mammals (wild and domestic animals, including marine mammals) proved susceptibility to *Leptospira* infection, however their sensitivity may differ from one serotype to another (host-serovar relationships), as well as between incidental and maintenance hosts (16). The severity of acute leptospirosis forms is influenced by the serotype (11). Although over 250 serotypes and about 21 genomospecies are known (18), only a small group of *Leptospira* serotypes proved to be associated with one or more maintenance hosts that serve as a source of infection and with increased prevalence within a particular geographic area (23). Leptospirosis occurs with a higher incidence in summer or autumn. A direct correlation between the large number of leptospirosis and the rainy seasons was reported in dogs (12), cattle (6), pigs (3), and humans (19). Also, *Leptospira* infection can have a high prevalence in shelters with inappropriate, unhygienic and overcrowded conditions (12, 13).

In dogs, the most common serovars are *L.* Grippotyphosa, *L.* Pomona, *L.* Icterohaemorrhagiae, *L.* Canicola, *L.* Autumnalis, *L.* Hardjo, and *L.* Bratislava (1, 4, 7, 10, 12, 24). The increased prevalence of these serovars was also reported in the Romanian dogs (2, 20, 25).

Leptospires are multiplied in the renal tubules, and then dispersed in the environment through contamina-

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ted urine. Leptospires can infect the host through the abrasions in healthy skin, mucous membranes or conjunctiva without lesions, lungs, after inhalation of aerosolized body fluid, and thus the exposure of humans to contaminated urine can lead to their infection (22).

The disease has a wide range of clinical manifestations, from mild subclinical infection to multiple organ failure and death (5, 16) and, for this reason, animals with unknown history that are introduced into shelter or other type of dog community should be assessed if they are *Leptospira* carriers. The natural hosts of some serotypes do not show clinical signs (asymptomatic forms), while some accidental hosts, including humans, can express severe disorders (e.g., renal failure, liver failure, pulmonary haemorrhages) (17).

After the acute disease, the dog can remain a carrier of *Leptospira*. Studies have shown that many dogs can be asymptomatic carriers, and they have furthered the spread of *Leptospira* in the environment through the infected urine (14, 15).

Effective control measures are avoiding contact between suspect and healthy animals, prophylactic quarantine for at least 30 days for the new dogs brought into a shelter, avoiding direct contact of people with infected animals and applying an effective vaccination program against leptospirosis throughout the unit (20).

In the context of several *Leptospira* serotypes circulation in the Romanian animal and human populations, in this study the following major objectives were established: (1) assessment of the *Leptospira* spp carriers in a dog shelter by PCR analysis of non-invasively collected urine samples; (2) the serotyping of *Leptospira* isolates in the targeted dog shelter; (3) assessing the usefulness of active surveillance of *Leptospira* spp infection in stray dogs by PCR analysis of non-invasively collected urine samples.

## MATERIALS AND METHODS

### Shelter and dogs included in study

The shelter has pens with capacity of maximum 10 dogs, where 102 stray dogs (Table 1) were accommodated in 20 pens (2-5 dogs /pen).

**Table 1**

Age and gender groups of dogs in the investigated shelter

| Gender | Age group | | | Total |
|---|---|---|---|---|
| | Youth | | Adults | |
| | < 6 months | 6–18 months | | |
| Males | 20 | 25 | 12 | 57 |
| Females | 15 | 10 | 20 | 45 |
| Total | 35 | 35 | 32 | 102 |

### Sampling, packaging and transportation of samples

Urine samples were collected in 15 pens (one sample /pen) by using a non-invasive cotton pads method previously developed (2, 25). Briefly, 5-8 ml of urine were collected, immediately or during the natural urination of the animals, with cotton pads (Cotoneve, Sisma, Italy), packaged in Whirl-Pak bags (Nasco, USA) and stored at 4–8°C up to 72 hours before testing.

For *Leptospira* serotyping, 19 blood samples were collected from animals housed in PCR-positive pens (Table 2). Blood samples collected from the cephalic vein or the lateral saphenous vein, in accordance with Directive 2010/63/EU of the European Parliament and of the Council. The samples were stored at 4–8°C and serologically tested the same day.

**Table 2**

Age and gender groups of dogs from which blood samples were taken to perform the MAT test for the diagnosis of leptospirosis in the investigated shelter

| Gender | Age group | | | Total |
|---|---|---|---|---|
| | Youth | | Adults | |
| | < 6 months | 6–18 months | | |
| Males | 3 | 5 | 4 | 12 |
| Females | 2 | 3 | 3 | 8 |
| Total | 5 | 8 | 7 | 20 |

### Method of *Leptospira* spp detection by Polymerase chain reaction (PCR)-based assay

The detection of *Leptospira* spp. in urine followed a method previously described by Baraitareanu et al. (2014) and Gurau and Dragan (2018) (2, 13), by using the Pure Link Genomic DNA Kits (Invitrogen, USA), in accordance with the manufacturer's instructions, Taq platinum polymerase (Invitrogen, USA), and oligonucleotide primers designed by Merien et al. (1992) (21).

In a nutshell, urine samples and controls were centrifuged 30 minutes at 15.000 rpm, eluted in 180µl Pure Link Genomic Digestion Buffer, mixed with proteinase K and heated 60 minutes at 55°C for. DNA Amplification (25 µl/reaction) used 0.5 µl polymerase (5.0 U/µL), 0.75 µl MgCl2 (1.5mM), 0.5µl dNTP solution (200µM) (Invitrogen, USA), 2.5µl PCR buffer (50mM KCl, 10mM Tris–HCl, pH 8.0), 0.5 µl of forward primer F (10 pmol), 0.5µl of reverse primer R (10 pmol) and 2.0 µl DNA extract from urine samples.

The following thermal cylce was used: denaturation - 3 minutes, 94°C; amplification - 1 minute at 94°C, 1.5 minutes at 63°C and 2 minutes at 72°C - 45 cycles; final extension: 10 minutes at 72°C (2, 13, 21).

**Method of *Leptospira* serovar detection by microscopic agglutination test (MAT)**

MATs were performed in accordance with OIE Terrestrial Manual, Chapter 2.1.9. Leptospirosis (26) by using the following serovars as source of antigens: *L.* Pomona, *L.* Ichterohaemoragiae, *L.* Canicola, *L.* Hardjo, *L.* Gripphothyphosa, *L.* Australis, *L.* Autumnalis, *L.* Bataviae, *L.* Ballum, *L.* Wollfi, *L.* Sejroe, *L.* Tarasovi.

## RESULTS AND DISCUSSIONS

The PCR-testing of urine samples revealed the presence of the specific DNA-sequence of *Leptospira* spp. in all pens with high-positive results (electrophoretic bands are clearly visible) in 13 tests, and low-positive results (the electrophoretic bands are discrete) in two tests (Table 3, Fig. 1).

**Table 3**

The results obtained by PCR testing of urine samples collected from the shelter for *Leptospira* spp.

| Results | Positive | Low positive | Negative | Total |
|---|---|---|---|---|
| Number of samples | 13 | 2 | 0 | 15 |

The MAT results revealed the concomitant circulation of three different *Leptospira* serovars (Table 4), *L.* Bataviae (18/19), *L.* Ballum (13/19), and *L.* Australis (1/19), but with high differences between the prevalence thereof (Table 5). Also, the MAT results revealed high-positive results (2+) in 29 tests, and low-positive results (<100) in three tests (Table 4).

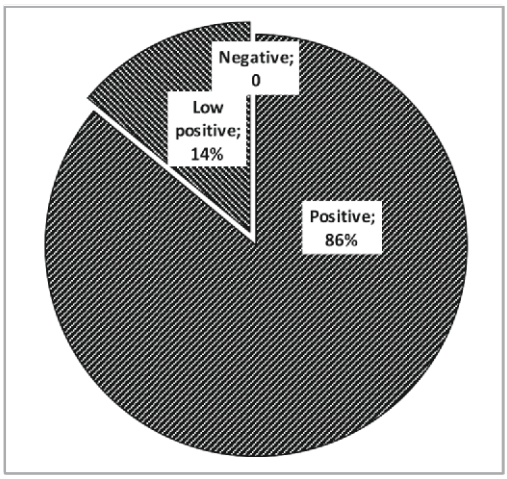


**Fig. 1.** Proportions of the results obtained by PCR testing of urine samples collected from the shelter

The results support the usefulness of the PCR test for *Leptospira* spp. surveillance of the street dogs housed in the community shelters by using a non-invasive method of the urine sampling with cotton pads.

**Table 4**

The results obtained by microscopic agglutination testing (MAT) of serum samples collected from the shelter for *Leptospira* serovar detection

| Serovar* | #1 | #2 | #3 | #4 | #5 | #6 | #7 | #8 | #9 | #10 | #11 | #12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Titer | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 | 1/100 |
| A 1 | N | N | N | N | N | N | N | 2+ | N | N | N | N |
| B 2 | N | N | N | N | N | N | N | 2+ | N | N | N | N |
| C 3 | N | N | N | N | N | N | N | 2+ | N | N | N | N |
| D 4 | N | N | N | N | N | N | N | 2+ | N | N | N | N |
| E 5 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| F 6 | N | N | N | N | N | 2+ | N | 2+ | 2+ | N | N | N |
| G 7 | N | N | N | N | N | N | N | 2+ | <100 | N | N | N |
| H 8 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| A 9 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| B 10 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| C 11 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| D12 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| E 13 | N | N | N | N | N | N | N | 2+ | <100 | N | N | N |
| F 14 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| G 15 | N | N | N | N | N | N | N | 2+ | <100 | N | N | N |
| H 16 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| A 17 | N | N | N | N | N | N | N | 2+ | 2+ | N | N | N |
| B 18 | N | N | N | N | N | N | N | 2+ | N | N | N | N |
| C 19 | N | N | N | N | N | N | N | N | N | N | N | N |

* #1 - L. Pomona; #2 - L. Ichterohaemoragiae; #3 - L. Canicola; #4 - L. Hardjo; #5 - L. Gripphothyphosa; #6 - L. Australis; #7 - L. Autumnalis; #8 - L. Bataviae; #9 - L. Ballum; #10 - L. Wollfi; #11 - L. Sejroe; #12 - L. Tarasovi

**Table 5**

*Leptospira* serovars prevalence in 19 serum samples collected from the dogs housed in PCR-positive pens

| Serovar | Positive/total samples | |
|---|---|---|
| | No. | % |
| #1 - *L.* Pomona | 0/19 | 0 |
| #2 - *L.* Ichterohaemoragiae | 0/19 | 0 |
| #3 - *L.* Canicola | 0/19 | 0 |
| #4 - *L.* Hardjo | 0/19 | 0 |
| #5 - *L.* Gripphothyphosa | 0/19 | 0 |
| #6 - *L.* Australis | 1/19 | 5.26 |
| #7 - *L.* Autumnalis | 0/19 | 0 |
| #8 - *L.* Bataviae | 18/19 | 94.74 |
| #9 - *L.* Ballum | 13/19 | 68.42 |
| #10 - *L.* Wollfi | 0/19 | 0 |
| #11 - *L.* Sejroe | 0/19 | 0 |
| #12 - *L.* Tarasovi | 0/19 | 0 |

This method is useful mainly if there is an unknown history of *Leptospira* infection in the shelter or in the catchment area. In a shelter, the initial testing of all newly arrived dogs by this non-invasive method of sampling, followed by MAT (gold standard methods for leptospirosis diagnosis) only in PCR-positive dogs, several risks and disadvantages of the classical protocol will be reduced. This will reduce the stress of restraint and the risk of human contamination. However, this working protocol has some limitations as well the determination of prevalence by PCR assays and of the renal-carriers.

In the researched shelter, the serological results revealed high *Leptospira* seroprevalence, which support the hypothesis of the high risk of human exposure to *Leptospira* infection, and consequently support the recommendation for the assessment of the workers in terms of leptospirosis infection. The increased seroprevalence of leptospirosis in dogs was also reported by Faine et al. (2000) but this study has not tested a close-contact dog population and the correlation of serological data with the prevalence of chronic leptospirosis was difficult because the serological titres were either small or negative (8).

## CONCLUSIONS

The specific DNA-sequence of *Leptospira* spp. was detected by PCR assays in 86% of the urine cotton pads sampled from a community shelter which housed 102 stray dogs. The microscopic agglutination test (MAT) has shown a 94.74% seroprevalence of *Leptospira* infection with 68.42% of dogs showing at least two *Leptospira* serovars inventions.

Three different serovars were identified in the shelter: *L.* Bataviae (94.74%), *L.* Ballum (68.42%) and *L.* Australis (5.26%). PCR assay and the urine-cotton pad sampling method can be effective tools for controlling and preventing infection in humans and other animals by periodically testing of stray dogs in the community shelters.

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