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Turkish Journal of Veterinary and Animal Sciences

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Research Article

Turk J Vet Anim Sci
(2015) 39: 719-723
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doi:10.3906/vet-1505-18

Seroepidemiological survey of leptospiral infection in stray dogs in Serbia

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Received: 06.05.2015 • **Accepted/Published Online:** 13.07.2015 • **Printed:** 31.12.2015

Abstract: Leptospirosis is a bacterial zoonosis with worldwide distribution. This disease is facilitated among stray dogs due to their lifestyle and the absence of immunoprophylaxis. The aim of the present study was to provide serological data on the presence of certain serovars of *Leptospira* spp., which are assumed to circulate in the population of stray dogs in Serbia. During a period of 3 years (from April 2010 to June 2013), 1045 canine sera originating from 11 shelters were submitted to the laboratory of the Department of Infectious Animal Diseases and Diseases of Bees, Faculty of Veterinary Medicine, Belgrade. A microscopic agglutination test (MAT) was performed to detect antibodies to *Leptospira* (cutoff, 1:100). The overall seroprevalence was 5.45% (57/1045) and the most prevalent *Leptospira* serovars were *Icterohaemorrhagiae* 33.3% (19/57), *Pomona* 29.8% (17/57), *Canicola* 14.0% (8/57), *Grippotyphosa* 3.5% (2/57), *Bataviae* 1.7 % (1/57), and *Sejroe* 1.7% (1/57). All dogs were seronegative for antibodies against serovars *Australis* and *Bratislava*. The results showed that stray dogs contribute to the spread and maintenance of *Leptospira* spp. in Serbia. Due to close contact with humans it is very important to improve the prevention of leptospirosis in dogs and support a One Health approach.

Key words: Leptospira, stray dogs, Serbia, serology, seroprevalence

1. Introduction

Leptospirosis is a bacterial zoonosis with worldwide distribution. The disease is caused by more than 200 different serotypes of the pathogen species *Leptospira interrogans* sensu lato (1). The most prevalent serovars associated with the disease in dogs are *Canicola*, *Icterohaemorrhagiae*, *Pomona*, *Bratislava*, and *Grippotyphosa* (2,3). In recent years, there has been an increase in the prevalence of leptospirosis and it has become a reemerging disease, probably due to changing infectious serovars (4).

Leptospira spp. serovars are maintained in the environment by mammalian reservoir hosts such as rats, mice, voles, and other small rodents (5). Dogs are an important factor in the occurrence of human infections because they act as an epidemiological link between reservoirs from the environment and people. By improving the prevention of leptospirosis in dogs, a One Health approach is supported (6).

The stray dog population suffers from this zoonosis more often than pet dogs do due to their lifestyle and the absence of immunoprophylaxis. Stray dogs may become infected by direct or indirect contact with mammalian reservoir hosts as a result of rummaging through garbage and hunting when searching for food, via water ingestion from puddles, by sniffing other animals' urine, licking the genital tract of females, and mating (7).

Infection in dogs may result in very variable symptomatology; while some dogs have mild or no signs of the disease, for others the illness can quickly become serious and can even cause death (8). Canine vaccination plays an important role in protection against *Leptospira*. Bivalent vaccines containing inactivated serovars *Icterohaemorrhagiae* and *Canicola* were developed in the 1970s (9).

The diagnosis of leptospirosis is very difficult due to its clinical complexity and can be done by the following methods: serological, polymerase chain reaction (PCR), fluorescent antibody testing of urine or tissue samples, or organism isolation (10). The most common diagnostic method used for the diagnosis of canine leptospirosis is the serological microscopic agglutination test (MAT) (2).

The government has been taking measures to solve the problem with stray dogs in Serbia for several years;

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the strategy includes placement of dogs in shelters and implementation of the catch–neuter–release program. Data regarding the health status of these dogs are very scarce. The aim of this seroepidemiological survey was to determine the seroprevalence of several *Leptospira* serovars in the population of stray dogs in Serbia. Based on the obtained results, we wanted to assess whether the current vaccine is sufficient to prevent the emergence of canine leptospirosis in Serbia in relation to the currently present serovars that circulate in the studied population.

2. Materials and methods

2.1. Serum collection

Whole blood samples of 1045 stray dogs originating from the territory of 11 municipalities in the Republic of Serbia were transported to the laboratory of the Department of Infectious Animal Diseases and Diseases of Bees, Faculty of Veterinary Medicine, University of Belgrade, during the 3-year period from April 2010 to June 2013. All samples were taken from dogs that had spent some time at municipal shelters. At the shelters, veterinarians performed castration/sterilization, vaccination against rabies, deworming and elimination of external parasites, but did not vaccinate against infectious diseases. Due to the lack of history data on the exact age of the examined dogs, an approximate age was determined by examining the teeth and the general physical condition at the time of blood sampling. Upon arrival at the laboratory, each blood sample was centrifuged, serum was collected and marked, relevant data were recorded (place of origin, sex of the animal, approximate age) and the samples were stored at –20 °C until analysis.

2.2. Serological examination

A MAT was carried out according to Office International des Épizooties-OIE manual (11). Live cultures of 8 *Leptospira* serovars were used as antigens: *Australis*, *Bataviae*, *Bratislava*, *Canicola*, *Grippotyphosa*, *Icterohaemorrhagiae*, *Pomona*, and *Sejroe*. Each sample that was positive in the screening test (first phase) in a 1:100 dilution was tested in the second phase to reach the endpoint dilution of antibody positive sera (dilutions of 1:300, 1:1000, 1:3000, 1:10,000, and 1:30,000) (12). For reporting purposes, whenever a serum gave a positive reaction on 2 or more serovars, the one with the highest titer was assumed as dominant.

3. Results

A total of 57 (5.45%) of the studied 1045 dogs had a positive MAT titer of 100 or higher for one *Leptospira* serovar. Among all seropositive sera, 37 (64.91%) were MAT positive to 1 serovar, as follows: *Pomona* (16/57, 28.1%), *Icterohaemorrhagiae* (11/57, 19.3%), *Canicola* (8/57, 14.0%), *Sejroe* (1/57, 1.7%), and *Batavie* (1/57, 1.7%). Fifteen sera (26.31%) were positive to 2 serovars and 5 sera (8.77%) agglutinated to 3 different serovars (Table 1). The titer on individual serovars ranged from 1:100 to 1:30,000.

The highest titers for *Leptospira* serovars were (in descending order): *Icterohaemorrhagiae* (19/57, 33.3%), *Pomona* (17/57, 29.8%), *Canicola* (8/57, 14.0%), *Grippotyphosa* (2/57, 3.5%), *Bataviae* (1/57, 1.7%), and *Sejroe* (1/57, 1.7%). Nine sera agglutinated in an equal titer to more than 1 serovar: *Icterohaemorrhagiae/Grippotyphosa* (6/57, 10.5%), *Icterohaemorrhagiae/Grippotyphosa/Canicola* (1/57, 1.7%), *Icterohaemorrhagiae/Canicola* (1/57, 1.7%), and *Pomona/Grippotyphosa* (1/57, 1.7%).

All dogs were seronegative for antibodies against serovars *Australis* and *Bratislava*. Serovar *Grippotyphosa* was present together with one (12/57, 21%) or two other serovars (5/57, 8.7%) (Table 1).

The majority of seropositive dogs (31/57, 54%) were female and 46% (26/57) were male. In terms of age, out

Table 1. The distribution of *Leptospira* serovars in seropositive dogs.

Multiple serovars	n	Seropositive					
		Serovar*	n	%	Serovar*	n	%
1	37	AU	-	-	GR	-	-
		BA	1	1.7	IC	11	19.3
		BR	-	-	PO	16	28.1
		CA	8	14.0	SE	1	1.7
2	15	IC+GR	12	21.0	IC+CA	1	1.7
		IC+PO	2	3.5			
3	5	IC+CA+GR	4	7.0	PO+GR+IC	1	1.7
Total:	57						

*AU-*Australis*; BA-*Bataviae*; BR-*Bratislava*; CA-*Canicola*; GR-*Grippotyphosa*; IC-*Icterohaemorrhagiae*; PO-*Pomona*; SE-*Sejroe*.

of all seropositive dogs, 18 (31.5%) were younger than 1.5 year, 27 (47.5%) were 2–3 years old, and 12 (21%) were older than 3 years (Table 2).

The survey included stray dogs originating from the territory of 11 city municipalities, located in 6 different regions in the Republic of Serbia.

As shown in Table 2, the largest number of tested dogs (514), and among them the largest number of seropositive dogs (34/57, 60%), originated from the city of Belgrade, meaning that the seroprevalence in the city was 6.61% (34/514). The highest seroprevalence was in Loznica (7/71; 9.86%) and the lowest was in Bujanovac, where all dogs were seronegative.

4. Discussion

A total of 57 (5.45%) sera samples out of 1045 samples were positive for *Leptospira* serovars. Since samples were collected from dogs that originated from 11 city municipalities and sampling was done randomly, we assume that our results indicate the real situation of canine leptospirosis and its distribution on the territory of the Republic of Serbia.

The most common serovars were (in descending order): *Icterohaemorrhagiae*, *Pomona*, *Canicola*, *Grippotyphosa*, *Bataviae*, and *Sejroe*. None of the dogs showed positive MAT reaction to serovars *Australis* and *Bratislava*. These results are consistent with our previous results (13) as well as with research conducted in the 1970s, when Trifunović et al. (14) tested 844 dog sera samples from the Belgrade territory. They found seroprevalence of 7.94% wherein the most seropositive samples were *Icterohaemorrhagiae*, *Canicola*, *Sejroe*, and *Pomona*. This suggests that the epidemiological situation regarding canine *Leptospira* infections in Serbia has not substantially changed over the last few decades.

Serovar *Icterohaemorrhagiae* had the highest prevalence. It is considered the most common causative agent of canine leptospirosis in Germany, France, Denmark, Croatia, Romania, Italy, and Greece (15). Our study confirmed that the situation in Serbia is similar, as 1/3 out of all MAT positive sera gave agglutination to this serovar in different titers. Such results are not surprising, considering the great number and the ubiquitous nature of rats, which are reservoirs for *L. icterohaemorrhagiae*. Vukićević et al. (16) reported that in the population of gray rats (*Rattus norvegicus*) from the Belgrade area, 82% of the harvested individuals were infected with this spirochete. Moreover, our current research is in accordance with the results of the epidemiological service, which revealed that the most cases of human leptospirosis in the Belgrade area were caused by *L. icterohaemorrhagiae* and *L. pomona* (17).

The second most common serovar was *L. pomona*. Its presence is recorded in dog populations in Hungary (18), Croatia (19), and Romania (20), all neighboring countries

Table 2. Number of examined and seropositive dogs, according to their origin, age, and sex.

City	Age			Sex		No. of seropositive dogs	Total examined
	≤1.5	2–3	≥3	M	F		
Pančevo	19	19	8	29	17	2	46
Leskovac	18	22	10	14	36	4	50
Loznica	20	46	5	37	34	7	71
Ub	16	28	7	17	34	1	51
Vršac	34	11	2	21	26	2	47
Bujanovac	NO	D	A	T	A	-	77
Požarevac	15	16	14	17	28	2	45
S. Mitrovica	36	38	17	36	55	1	91
Šabac	19	2	1	11	11	2	22
N. Pazar	23	4	4	19	12	2	31
Beograd	188	67	37	93	255	34	514
Total:	388	253	105	294	508	57 (5.45%)	1045
	(37%)	(24%)	(10%)	(28%)	(49%)		
	no data for 29%			no data for 23%			

of Serbia. Contrary to reports from many European countries, which hardly ever mention *Pomona*, in the United States this *Leptospira* serovar is more often identified as the cause of clinical cases of canine leptospirosis (21).

Serovar *Canicola* is maintained by dogs and has no other known maintenance host. The consensus view is that the seroprevalence of *Canicola* is decreasing in many European countries (22); that is attributed to the use of vaccines for the past half a century. The fact that 14% of seropositive dogs in the present study had relatively low titers (1:100–1:300) on *L. canicola* indicates that this serovar is still actively circulating among dogs in Serbia. However, serological evidence about the dogs' exposure and occasional clinical cases caused by *L. canicola* still exists (15).

It is interesting that in the current study there were no positive sera samples to *L. bratislava*; this is contrary to numerous European and US research results, which recorded very high seroprevalence of this serovar in dog populations. *Leptospira bratislava* is very common in dogs in Switzerland, Great Britain, Germany, and Italy (15); in France it is the most common of all serovars with seroprevalence of 21.86% (23), and it is also present in the United States, where it is the second most frequent, just after serovar *Grippotyphosa* (24). We have detected serovar *Grippotyphosa* only in combination with one or two serovars, whereas it was dominant in the titer in only 2 dogs.

It is well known that dog serum can react with several *Leptospira* serovars. The general assumption is that a serovar that has the highest titer is the causative agent of infection (2,3,24). However, out of 57 MAT positive sera in our trial, 9 of them agglutinated with 2 or 3 serovars (that belonged to different serogroups) in equal titers: 6 sera had equal titers to *Icterohaemorrhagiae/Grippotyphosa* (1 × 1:100; 2 × 1:300; 1 × 1:1000; 2 × 1:3000); one serum had 1:300 titer to 3 serovars, *Icterohaemorrhagiae/Grippotyphosa/Canicola*; one serum had 1:100 titer to *Icterohaemorrhagiae/Canicola*; and in one serum, serovars *Pomona/Grippotyphosa* had equal titers at 1:300. In all cases, the equal titers are probably the result of coagglutination, although there was a possibility that the infection was caused by more than one serovar. In order to reveal which serovar is the infective one, *Leptospira* isolation should be done instead of serology; unfortunately, this method is time- and money-consuming and it is very rarely done (25). Thus, although it is simple and easy to do MAT, this is a subjective method that relies on many factors such as the terms of cultivation and the quality of live *Leptospira* cultures used as antigens in the reaction, as well as on the lab technicians' experience (26).

Our results clearly indicate that stray dogs placed at shelters throughout Serbia are potential maintainers of this zoonotic disease and contribute to the spread and maintenance of *Leptospira* spp. in Serbia. As the global control of canine leptospirosis is not possible through control of the natural sources of infection, vaccination is still the best method of disease prevention. Since crossimmunity between different *Leptospira* serovars does not exist or is very low (27), there is a need for inclusion of circulating serovars in the vaccine. Therefore, the enhancement of dog protection against leptospirosis in Serbia may be obtained by an update of the anti-*Leptospira* vaccine with the inclusion of some new components such as serovar *L. pomona*. In addition, veterinary practitioners should encourage dog owners to constantly vaccinate their dogs. The vaccination against infectious diseases is not obligatory by law in Serbia and many owners do not do it, although they should due to the epidemiological situation.

Acknowledgment

This study was financially supported by the Ministry of Education, Science, and Technological Development, Serbia, grant TR 37015.

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