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Canine leishmaniasis prevalence in the Slovenian dog population

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Abstract

Introduction: Leishmaniasis is a life-threatening zoonosis of which dogs are the major reservoir and sandflies are the vectors. Until now, the prevalence of canine leishmaniasis (CanL) in the Slovenian dog population was unknown. **Material and Methods:** Epidemiological data, eye swabs and blood samples were taken from 465 dogs born in Slovenia and older than one year. Commercial ELISA kits and real-time PCR were used. For ELISA-positive samples, an immunofluorescence antibody test (IFAT) was performed. Descriptive statistics were used to characterise the samples. The one-sample nonparametric chi-square test was used to test whether the categories of a variable were equally distributed. **Results:** A 59.9% proportion of the recruited dogs had travelled to endemic regions and 62.1% of them had not been protected by insect repellents. Skin symptoms that might be CanL-related were described in 109 of the dogs' histories (23.4%), inappetence and/or weight loss in 25 (5.4%), and anaemia, intermittent fever, and/or lymphadenopathy in 19 (4.1%). At the time of recruitment, all dogs were asymptomatic. All samples were PCR negative, nine (1.9%) were ELISA positive, but none were IFAT positive. Five of the nine ELISA-positive dogs were non-travellers. **Conclusion:** We conclude that the seroprevalence of canine leishmaniasis of 1.9 % in the autochthonous Slovenian dog population may pose a risk of endemic spread of the disease.

Keywords: canine leishmaniasis, CanL, prevalence, vector-borne diseases, dogs.

Introduction

The visceral form of human leishmaniasis (HumL) is a life-threatening disease that affects 200,000–400,000 people worldwide annually. It has the second highest mortality rate among parasitic diseases in humans with an estimated 20,000–40,000 deaths per year (2, 30). Leishmaniasis is also of great importance in veterinary medicine, as infection with *Leishmania infantum* can cause a severe, potentially fatal disease in dogs. If not diagnosed early, dogs lose weight heavily and exhibit intermittent fever, generalised lymphadenopathy, and exfoliative dermatitis. The most common cause of death or reason for euthanasia in affected dogs is renal failure (3). In addition, dogs are considered the main reservoir of these parasites for humans (12). Veterinarians must therefore aim to achieve two goals: first, to treat animal patients with canine leishmaniasis (CanL) in accordance with the latest treatment guidelines (30); and second, to prevent or minimise transmission from infected dogs to vectors and, consecutively, to other animals and humans.

Leishmaniasis is endemic in the Mediterranean region. Traditional endemic areas include Italy, Spain, Portugal, Greece, Bulgaria, Croatia and France (7), with Italy and Croatia bordering Slovenia. The endemic area has recently expanded towards northern Europe (27), and sandflies have recently been detected in regions previously mapped as free of the disease (14, 15, 32). A high seroprevalence of CanL (31%) was found in Dalmatia, a region of Croatia (35), while the overall seroprevalence in Croatia was recently reported to be 1.38% (22). Slovenia should not yet be considered an endemic region. Recently, updated data on HumL and CanL cases and the presence of competent *Phlebotominae*

fly vectors in Slovenia have been reported. The same source also indicated an increase in the number of infected dogs that have been adopted in Slovenia (16). The presence of endemic areas in neighbouring countries (Italy and Croatia), an increased number of imported infected dogs and the simultaneous presence of competent *Phlebotominae* fly vectors in a climatically suitable region may lead to endemic spread of the disease. Therefore, the aim of the study was to determine the current prevalence of leishmaniasis among autochthonous dogs in Slovenia as a country with currently undetermined CanL status bordering endemic countries.

Material and Methods

As part of the study, a guide for the practical handling of leishmaniasis-infected dogs was prepared by the members of this project for practicing physicians in Slovenia. To inform dog owners, an article about leishmaniasis was published in a local newspaper with an invitation to participate in the study. The invitation was also published on the website of the Faculty of Veterinary Medicine of Ljubljana. Pet owners were informed by the participating veterinarians about the necessary preventive measures.

Dogs. Thirty private veterinary practices evenly distributed across the country were recruited to participate by collecting samples. Eye swabs and blood samples were collected from 465 dogs from April to October 2018, as statistically planned in advance according to a binomial equation. We considered the whole dog population to be 225 884 deriving from the total of all registered dogs in Slovenia in February 2018 and 5% the response distribution (estimated based on precedents from other researchers) and decided to accept a 2% margin of error and tolerate a 95% confidence level. On this basis, 465 dogs were proportionately included from 10 regions (based on population data). There were two inclusion criteria: the dogs had to have been born in Slovenia and should have been at least one year old. Informed consent was provided by the dog owners for each sample, and a health status and travel history questionnaire was completed by each owner.

PCR. A real-time PCR was performed on eye swabs and blood using a genesig Leishmania Standard Kit (Primerdesign™ Ltd, Chandler's Ford, UK).

Serological analysis. Blood samples were tested using a commercial VetLine Leishmania ELISA kit (ref. LEIVT0310, Novatec, Dietzenbach, Germany), which uses native *L. infantum* antigens. For this analysis, promastigotes were grown on Roswell Park Memorial Institute/Serum Fetal Bovine (RPM/SFB) and a crude extract was harvested. As the ELISA test is semi-quantitative, the results were expressed in units (<9 units – negative, 9–11 units – borderline, >11 units – positive). Blood samples from nine dogs that gave positive results with the ELISA kit were collected again in February 2019 and sent to the World Health Organization (WHO) Collaborating Centre for leishmaniasis, the Institute Health Carlos III (ISCIII), Majadahonda, Madrid, Spain. They were tested by in-house indirect fluorescent antibody test (IFAT). Sera were tested in serial twofold dilutions from 1/10 to 1/640 which were applied to slides containing acetone-fixed *L. infantum* promastigotes and incubated at 37°C for 30 min. After washing three times in phosphate-buffered saline, antibody fixation was revealed with fluorescein isothiocyanate-conjugated sheep anti-dog IgG (ICN, Aurora, OH, USA) diluted to 1/150 in 0.01% Evans blue for counterstaining. Slides were incubated at 37°C for 30 min, washed and examined with a fluorescence microscope. The titre corresponds to the final dilution at which at least 50% of the parasites have visible fluorescence. Dogs were considered infected when the titre was ≥1/160.

Statistical analysis. All statistical tests were performed using SPSS Statistics 21 (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to characterise the sample. The nonparametric one-sample chi-square test was used to test whether the categories of a variable were equally distributed, and a P value of <0.05 was considered statistically significant.

Results

Blood and eye swab samples were collected from 465 dogs of both sexes. The dogs' epidemiological data are presented in Table 1. Various breeds were represented among the dogs sampled (Table 2).

Table 1. Dogs' epidemiological data

		Number of dogs tested	% of dogs tested
Gender			
	male	247	53.1
	female	218	46.9
Age			
	1 to 3 years	111	23.9
	>3 to 7 years	188	40.4
	≥ 8 years	166	35.7
Travel			
	outside Slovenia	278	59.8
Insecticide use			
	repellents	107	23.0
	non-repellents	254	54.6
	both	38	8.2
	none	35	7.5
	undefined	31	6.7

The median age of the dogs involved was 6 years (minimum 1 year, maximum 18 years), and the age distribution was 111 (23.9%) 1–3 years old, 188 >3–7, and 166 (35.7%) ≥8 years old. Almost two-thirds of the dogs (59.8%) had travelled to endemic regions at least once in their lives. Many of them (289; 62.1% of the recruits) were not protected with an insect repellent (Table 1). None of the included dogs were vaccinated against leishmaniasis.

Owners were asked about any history of skin problems, anaemia, lymph node enlargement, recurrent high body temperature, anorexia, or weight loss in the dogs. The history of 126 (27.1%) of the included dogs disclosed symptoms that could correspond to CanL (Table 3). The majority (109 dogs) had exhibited skin symptoms. Twenty-one (4.5%) showed a combination of symptoms (Table 3). Three dogs had been diagnosed with leishmaniasis in the past, treated prior to inclusion, and had negative results in all tests at the time of the study. ELISA was performed on all 465 blood samples from the dogs. Nine (1.9%) showed a positive ELISA result (Table 4). These dogs were re-sampled in February 2019 and additionally tested by IFAT, in which all 9 yielded a negative result. None of the 465 dogs included were PCR-positive by either eye swab test or blood test.

Table 2. Breeds of the recruited dogs

Breed	Number	Breed	Number	Breed	Number	Breed	Number
Mixed breed	133	Shih-tzu	5	Pug	2	Kerry blue terrier	1
Golden retriever	31	Beagle	5	Jack Russell terrier	2	Chinese crested	1
Labrador retriever	22	Newfoundland	4	Weimaraner	2	Short-haired dachshund	1
German shepherd	21	Australian shepherd	4	Saint Bernard	2	Lagotto Romagnolo	1
Boxer	19	Rottweiler	4	Irish soft-coated wheaten terrier	2	Toy poodle	1
Border collie	18	Chihuahua	4	Caucasian shepherd	2	German wirehaired pointer	1
German spaniel	12	Vizsla	4	Great Dane	2	Parson Russell terrier	1
American Staffordshire terrier	11	Pekingese	4	Entlebucher	2	Pit bull terrier	1
Cocker spaniel	11	Whippet	4	Schnauzer	2	Montenegrin mountain hound	1
French bulldog	9	Airedale terrier	3	King Charles spaniel	2	Rhodesian ridgeback	1
Bernese Mountain Dog	8	Beauceron	3	chow chow	2	White Swiss shepherd	1
Alaskan Malamute	7	Miniature pinscher	3	Lakeland terrier	1	Welsh corgi	1
West Highland terrier	6	Dobermann	3	Black Russian terrier	1	Hovawart	1
Samoyed	6	Rough collie	3	Landseer	1	Havanese	1
Tibetan terrier	6	Siberian husky	3	Maltese	1	Croatian Shepherd	1
Greater Swiss mountain dog	6	Brandl-bracke	3	English springer spaniel	1	Irish red setter	1
Yorkshire terrier	5	Malinois	3	Argentine mastiff	1	Istrian coarse-haired hound	1
King Charles Cavalier	5	Flat-coated retriever	3	Bordeaux mastiff	1	Undefined breed	3
Dalmatian	5	English bulldog	2	Coton de Tuléar	1		
Dachshund	5	Poodle	2			Total	465

Table 3. Disease symptoms commensurate with canine leishmaniasis from the history of dogs included in the study

Past symptoms	Number	%
None commensurate with canine leishmaniasis	339	73.1
symptoms that could correspond to CanL	126	27.1
[a]skin problems	109	23.4
[b]anorexia or loss of weight	25	5.4
[c]anaemia or lymph node enlargement or recurrent high body temperature	19	4.1
a & b	11	2.4
b & c	3	0.6
a & c	2	0.4
a & b & c	5	1.1

Table 4. Dogs with positive ELISA results

Dog identification number	ELISA result	History of travelling	Ectoparasiticide use	History of symptoms corresponding to CanL	Breed
266	11.44 NTU	None	Frontline	None	Mixed
265	12.37 NTU	None	Frontline	None	German shepherd
225	11.60 NTU	None	Nexgard	None	Caucasian shepherd
215	11.68 NTU	None	No ectoparasiticide	None	Mixed
204	12.74 NTU	SRB, CRO, A	Nexgard	None	German wirehaired pointer
191	13.71 NTU	CRO	Foresto	Skin	Mixed
190	12.38 NTU	CRO	Nexgard	None	Shih-tzu
186	13.44 NTU	None	Frontline	Skin	German shepherd
172	22.30 NTU	CRO, ITA, HUNG, A	Bravecto	None	Border collie

CanL – canine leishmaniasis; SRB – Serbia; CRO – Croatia; A – Austria; ITA – Italy; HUNG – Hungary

Discussion

CanL affects approximately 2.5 million dogs annually in the Mediterranean region (21). Reports of seroprevalence in different countries in Europe vary and are highly dependent on the geographical and climatic conditions in a given part of the country. The average seroprevalence of CanL is 8.5% in Spain, 20.0% in Portugal and Cyprus, 4.0–20.0% in Southern France, 2.0–15.0% in Italy, 25.0% in Greece, 15.7% in Turkey (7) and 1.4% in Croatia (22).

The sexes of the dogs involved were statistically equally distributed (χ^2 =1.809, df = 1, P = 0.179). None of the included dogs were representatives of known resistant breeds (e.g. Ibizan dog) and 55 dogs (11.8%) were representatives of susceptible breeds (21 German shepherds, 19 boxers, 11 cocker spaniels and 4 Rottweilers) (6, 10, 28, 29).

According to the literature, the distribution of the disease is bimodal with the highest prevalence in dogs younger than 3 years and older than 8 years (13). Our dogs were distributed by age as shown in Table 1, with 59.9% in either one or the other age range with the highest predilection to infection.

Preliminary studies showed that infected dogs were adopted from endemic regions and settled in Slovenia (16). Concern for the risk of spread of the disease from neighbouring endemic regions, as well as the possibility of endemic spread motivated the decision to conduct the present study. It was decided to include dogs older than one year and born in Slovenia. The reasons for this decision are explained as follows. It is known that a certain number of infected dogs and a certain number of competent vectors in an area are necessary for the endemic spread of the disease (13, 25). Unfortunately, entomological studies of phlebotomine vectors in the region are sparse (14). The criterion of including only dogs born in Slovenia was therefore set in order to reveal the endemic spread of the disease should positive reactors be found without any travel in their history. The second reason was to avoid the deliberate inclusion of sick dogs recently imported from endemic regions. During the preliminary studies, it became apparent that the collaborating veterinarians wanted to take advantage of free diagnostic evaluation for their clinical patients recently imported from Bosnia. This would bias the epidemiological study. We therefore decided to conduct the study in such a way as to enable us to find endemic infection using the history of the included dogs if they were positive in tests. In this case, it was decided that we would focus on dogs that had not travelled inbound to Slovenia.

At the time of the study, the dogs involved were clinically healthy. In the past, 27.1% of them had shown symptoms that could correspond to CanL (Table 3). The most common clinical manifestations of CanL include skin lesions with or without nail deformation (onychogryphosis), generalised lymphadenomegaly, loss of body weight, and intermittent fever. One of the most common laboratory abnormalities in CanL is nonregenerative anaemia (3, 30). We included these clinical signs in a questionnaire completed by the dog owners in our study. The observed symptoms were attributed to other diseases in all but three dogs. The medical history of these three dogs revealed that they had been previously diagnosed with CanL, and two of them had been treated. These three dogs had travelled to endemic regions (one to Italy, one to Croatia, and one to Italy, Austria, France, Switzerland, and the Czech Republic). At the time of our study, all three dogs were clinically in remission and showed antibody titres below the limit of detection.

The real-time PCR results of the eye swabs and blood in our study were all negative. The specificity of molecular tests is typically 95% to 100%. One limitation is the sample source, which has a significant impact on the ability of molecular tests to identify infected dogs. Bone marrow and lymph node samples yield the highest number of positive results compared to other sites, regardless of clinical status (31). Nevertheless, collection of bone marrow and lymph node puncture biopsies is not suitable for epidemiological studies due to its invasive nature. Blood samples are easy to obtain but are not among the best tissues for PCR testing because the parasites are not constantly present in the bloodstream (30). In addition to blood samples, eye swabs were also collected in our study. According to current literature, they are considered good material for PCR (8, 17, 31). In 2014, Ceccarelli *et al.* (5) showed that the sensitivity and specificity of PCR when testing conjunctival swabs were 87% and 96%, respectively. Collection of eye swabs may in some cases have been from dogs carrying *Leishmania* but asymptomatic. This is a limitation to this study because it may have affected the total of negative PCR results; an underestimation of the presence of *Leishmania* may have resulted.

Many of the dogs involved in our study (59.8%) had travelled to endemic regions at least once in their lives. Not all of them were sufficiently protected, either by effective repellents or by vaccination. Nevertheless, all but three remained healthy. This does not necessarily mean that they were not infected. We know that dogs can remain healthy if exposure to infected vectors is brief (e.g. during a vacation in an endemic area), if parasitic invasion is at low level, and if they are immunocompetent. The parasites may remain confined to the site of inoculation (skin) or to regional lymph nodes. We know that the majority of infected dogs never show symptoms (30).

Nine out of 465 blood samples gave low positive results with the ELISA test (Table 4). Our decision to perform an additional IFAT test was made on the basis that IFAT was considered a good standard test for CanL in non-endemic areas at the time of the study (1). However, considering current references, IFAT proves to be the best test only for symptomatic dogs and not for asymptomatic individuals. The sensitivity of IFAT in asymptomatic dogs may be only 29.4-31.25% (1, 18). Because all dogs were asymptomatic at the time of

recruitment, it is plausible that IFAT showed negative results in dogs with low positive titres determined by ELISA. In addition, sera tested with IFAT were collected later than those tested with ELISA. The collection was performed in February 2019. There is a high likelihood that the antibody titres of dogs that were positive by ELISA test in October 2018 fell below the threshold during the 2019 winter season. As shown in the study on levels of antibodies to phlebotomine salivary antigens, they are known to fluctuate seasonally. They rise in the spring and fall below detection levels in the winter. The canine humoral response to phlebotomine antigens correlates with the annual dynamics of sandfly activity expected for the region. Significantly lower IgG levels are observed during the non-transmission season (33). Since cross-reactions and false positives must be considered in any serological test, ELISA is considered the most sensitive method in our case. The sensitivity and specificity of the ELISA used in our study were 95.8% and 95.43%, respectively, according to the manufacturer's validation. The low titre positive results obtained in nine samples in our study should therefore correspond to infected but healthy dogs. According to the literature, low antibody levels are characteristic of subclinical infections or exposed but uninfected dogs (31). The majority of our ELISA positives (7 / 9) had no symptoms corresponding to CanL in their medical history. Five out of nine positives had never travelled abroad. Therefore, the infection or exposure must have occurred within Slovenia. Various routes of infection have been demonstrated in dogs (the placental route, through mating, through blood transfusion, and through infected sandfly bites) (30). Sandflies were recently detected in Slovenia by Ivović *et al.* (14), and to the authors' knowledge, that research is the only entomological study which has been conducted for this area. We do not know the actual occurrence or probable infectivity of sandflies in our country. However, the climate appears to be suitable as a sandfly habitat. Flies have also been detected in European countries north of Slovenia, such as Austria (26) and Germany (24). Detection of anti-sandfly salivary antibodies in the blood of dogs can be used as a marker of exposure to *L. infantum* vectors (33) and may help to distinguish between false-positive reactors and dogs with contact.

There are three main forms of human leishmaniasis: visceral, cutaneous, and mucocutaneous, ranging in severity from cutaneous lesions resolving without treatment through debilitating mucocutaneous infections to life-threatening visceral disease (4). Human visceral leishmaniasis is characterised by irregular episodes of fever, weight loss, hepatosplenomegaly and pancytopenia due to replication of *Leishmania* parasites in macrophages mainly in the liver, spleen and bone marrow (20). In the Mediterranean region, visceral leishmaniasis is caused by *Leishmania infantum* and is considered one of the major opportunistic infections in patients with HIV (20).

According to data in the literature, the prevalence of human leishmaniasis correlates directly with the number of infected dogs in a region (9). In the WHO European region, most (almost 75%) of the human visceral leishmaniasis cases are reported from Albania, Georgia, Italy and Spain. Visceral leishmaniasis is endemic in nine countries of the European Union – Bulgaria, Croatia, Cyprus, France, Greece, Italy, Malta, Portugal and Spain. Of these, Italy and Spain have the highest annual estimated incidence of 160–240 and 140–210 cases, respectively, followed by Greece with an annual estimated incidence of 50 to 80 (2, 11, 34). In Croatia, the annual incidence is estimated to be 6–8 (2).

Recent reports of human leishmaniasis (HumL) cases in our country show low numbers. From 2010 to 2018, five cases of HumL were reported to the Slovenian National Institute of Public Health, including three cases of cutaneous leishmaniasis and two cases of the visceral infection. All of them were imported cases (23). The two patients with visceral leishmaniasis and one with the cutaneous type were infected in the Dalmatia region of neighbouring Croatia. Due to the low number of HumL cases, low seroprevalence in dogs in our country was expected. Nevertheless, global warming causing the northward spread of leishmaniasis vectors in Europe and importation of infected dogs into the country are two factors that increase the risk of establishing a new autochthonous transmission site for the parasite. The present work provides the basis for a national surveillance programme that should monitor the presence of canine and human cases of leishmaniasis in order to prevent the spread of the disease in Slovenia. Simultaneous concern for animal and human welfare is recommended by the World Health Organization in the One Health approach. Treatment of dogs may not completely eliminate parasites from the body, but it can significantly reduce the infectivity of treated dogs to vectors (19). Veterinarians must therefore treat canine patients with leishmaniasis in accordance with the latest treatment guidelines for the disease (30) and also educate dog owners about this threatening disease and its prevention measures.

We conclude that the leishmaniasis seroprevalence in autochthonous Slovenian dogs is 1.9%. Five out of nine positive dogs had never travelled out of the country, which is a circumstance suggestive of the endemic spread of the disease. This should be confirmed in future studies.

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