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

SEROLOGICAL STUDY OF EXPOSURE TO *LEISHMANIA* IN DOGS LIVING IN SHELTERS, IN SOUTH-EAST ROMANIA

STUDIU SEROLOGIC AL EXPUNERII LA *LEISHMANIA* LA CÂINII CARE TRĂIESC ÎN ADĂPOSTURI, ÎN SUD-ESTUL ROMÂNIEI

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ABSTRACT

One of the most important canine vector-borne diseases in the Mediterranean region is canine leishmaniosis and there are frequent reports that it is spreading to the North and East of Europe. Romania is one of the countries traditionally considered non-endemic. However, *L. infantum* infections in dogs, humans and one jackal, as well as vector-competent sandflies have been recently reported in the country. To determine the seroprevalence of this infection we collected sera from 110 stray dogs, from two counties (Galați and Călărași), from the south-east of Romania and investigated for the presence of IgG antibodies against *Leishmania* by an in-house ELISA. Anti-*Leishmania* antibodies were detected in 5 stray dogs from the county of Galați. The prevalence in the county of Galați was 8.33% and the overall prevalence was 4.54%. These results are comparable to what was reported before in the southern county of Vâlcea and suggest that stray dogs from the south of Romania are exposed to *L. infantum*. There is a need for more studies in order to understand the situation of *L. infantum* infections in Romania.

Keywords: *Leishmania,* Romania, serology, stray-dogs

REZUMAT

Leishmanioza canină este considerată una dintre cele mai importante boli vectoriale ale câinelui în țările din zona mediteraneeană. În ultimii ani, această boală este întâlnită frecvent la Nord și Est de zona endemică din sudul Europei. România este una dintre țările considerate non-endemice, dar unde au fost raportate recent infecții cu *L. infantum* la câine, om și șacal.

În România au fost găsiți și flebotomi capabili să transmită *L. infantum*. Pentru a determina seroprevalența leishmaniozei, am colectat ser de la 110 câini comunitari, provenind din două județe (Galați și Călărași), din sud-estul României și am detectat prezența anticorpilor IgG anti-Leishmania, folosind tehnica ELISA. Anticorpi anti-Leishmania au fost detectați la 5 câini din județul Galați. Raportăm o seroprevalență totală de 4.54% și o seroprevalență în județul Galați de 8.33%. Aceste rezultate sunt comparabile cu seroprevalența raportată în trecut, în județul Vâlcea și arată că, în sudul României câinii comunitari sunt expuși la infecții cu *L. infantum.*

Cuvinte cheie: *Leishmania,* România, serologie, câini fără stăpân

Canine leishmaniosis (CanL) is a zoonotic disease caused by parasites of the genus *Leishmania* and it is endemic in over 70 countries from all continents, except Oceania (4, 6).

Although several species of *Leishmania* can infect dogs, *Leishmania infantum* is the most important agent of CanL and the most widely spread species in Europe, where it is transmitted by the bite of infected female sandflies of the genus *Phlebotomus (17)*.

Leishmania infantum is endemic in Mediterranean countries but as for other vector-born diseases, recent studies show a change in its distribution that is attributed to climate change and other man-made environmental changes (10, 15). Travelling of companion animals also play an important role in the introduction of vector-borne diseases in non-endemic areas (5, 9).

This route of expansion in new areas is particularly relevant in the case of *L. infantum* infections because the dog represents the main reservoir for this disease and dogs travelling between Mediterranean countries and Romania are very common (9, 17).

This change in the distribution of CanL in Europe is well documented. There are examples of CanL present in areas previously considered non-endemic from Italy (15) and Spain (11). In eastern Europe, the situation is similar, as autochthonous CanL cases have been re-

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cently reported in countries considered to be non-endemic, like Bulgaria (19), Hungary (18) and Romania (6, 12) and *L. infantum* infected sandflies have been reported in Serbia (20).

In areas of south Romania both human and canine leishmaniosis cases have been reported between 1912 and 1969, while there is a gap in reports between 1969 and 1998 (3, 12). This lack of human and canine leishmaniosis cases coincides with the drastic fall in the incidence of malaria cases that was reported after the intense use of dichlorodiphenyl trichloroethane (DDT) and hexachlorocyclohexane (HCH) between 1949 and 1954 as part of the malaria eradication campaign (12, 14). It is reasonable to assume that the lack of reported cases of leishmaniosis was a welcomed consequence of the malaria eradication campaign.

After 1989, the number of people and companion animals travelling between Romania and South European countries increased because of the fall of the communist regime in Romania. This trend only increased after Romania joined the European Union. As a consequence, reports of people and dogs infected with *L. infantum* in Romania, after travelling to endemic areas became quite common (7, 16). The spread of *L. infantum*, with travelling dogs from endemic countries is a plausible scenario, considering that sandflies are still reported in Romania (1, 2, 9).

While autochthonous human or CanL cases are still not common occurrences in Romania, between 2013 and 2017 there have been several reports of autochthonous *L. infantum* infections in dogs, one human and a jackal (*Canis aureus*) (6, 7, 12, 13).

Either because of climate changes or because of changes in travelling patterns, there is a real risk that leishmaniosis is re-emerging in Romania but there is a need for more studies in order to assess this risk.

Considering the importance of CanL for veterinary medicine, the role of reservoir that dogs play for *L. infantum* but also the possible impact on human health, the objective of this study is to assess the seroprevalence of CanL in the south-east of Romania.

MATERIAL AND METHODS

Animals and sampling locations

Serum samples were collected between the 9th and the 11th of August 2017 from 110 dogs living in two municipal shelters from the south-east of Romania (Fig. 1), one near the city of Galați (45°27'14.1"N 28°04'34.0"E) and one near the city of Călărași (44° 12'28.7"N 27°19'52.3"E).

Fig. 1. Map of Romania and the two sampling locations. A blue star marks the location of the cities where the shelters are located. The counties where the cities are located are highlighted and have the same name as the cities.

The dogs in the shelters, were captured from the corresponding city, outskirts and nearby villages within a period of two weeks before the sample collection. All samples were collected by cephalic venipuncture, in tubes with clot activator. The sera was obtained by centrifugation of the clotted blood and was stored at -20°C until used.

The sampled dogs received no antiparasitic or prophylactic treatment and the sampling was performed with the consent of the shelter management.

ELISA test

The antigens used in the assay were prepared as previously described (8). Checkerboard titration was used to determine the optimal antigen concentration at 2.5 mg/ml. Polystyrene micro-ELISA plates were coated with 100 ml/well of soluble antigen, in 0.1M carbonate buffer (pH 9.6) and were left overnight at 4°C. The wells were washed three times with distilled water containing 0.05 Tween 20 (Sigma) and coated with the sera diluted at a concentration of 1/300 in PBS (pH 7.2).

The serum samples were tested in duplicate, on the same plate, with three positive and three negative controls. After a 3 h incubation period, at room temperature, another three washes were performed and 100 ml of the secondary antibodies (Anti-Dog IgG (whole molecule)-FITC antibody, F7884, SIGMA®) 1:30.000 in PBS, were added to each well and the plate was incubated as described above. After a final wash p-nirophenyl phosphate (SIGMA®) diluted in

carbonate buffer, pH 9.8 (1 mg/ml) was added and the plate was incubated for 20 min at room temperature.

The reaction was stopped using 50 mlNaOH 1N and optical densities were measured with a 405 nm measurement and a 630 reference filter, using the microplate reader HUMANREADER (HUMAN Diagnostic Systems, Germany).The cut-off value was determined by the mean of negative controls plus three standard deviations(8).

Statistical analysis

The prevalence and it's 95% confidence intervals were calculated using the Epi Info™ 7.2 software, provided by the Center for Disease Control (CDC, Atlanta, USA). The differences of prevalence each sampling location was analyzed using the Pearson Chi-square test, IBM SPSS Statistics 20.0 software.

RESULTS AND DISCUSSIONS

Five out of 110 serum samples were positive for anti-*Leishmania* spp. IgG (4.54%) (Table 1). The positive dogs were all found in Galați. The difference in prevalence between the two sampling locations was not statistically significant.

Table 1

The prevalence of *L. infantum* in each sampling location

County	City	Pos/tot	Prevalence	95% CI
Călăraşi	Călăraşi	0/50	0%	-
Galaţi	Galaţi	5/60	8.33%	0.027-0.183
Total:		5/110	4.54%	0.014-0.102

Recent epidemiological studies on canine leishmaniosis in Romania are scant. Historical studies published between 1912 and 1955 reported autochthonous cases of human and CanL and even an outbreak of human leishmaniosis in children, in the south of the country, in Dolj county (3, 12). Individual cases of human leishmaniosis were also reported in the south of Romania, in the counties of Giurgiu and Prahova (3, 12). Epidemiological studies reported in 1967 and in 1968 showed a prevalence of 1.2% and 2.2% of *L. infantum* infections in dogs from the southern counties of Dolj and Caraș-Severin(12).

Phlebotomus neglectus and *P. perfiliewi* are both incriminated vectors of *L. infantum* and have been reported between 1910 and 1971, in regions from south of Romania such as Dobrogea, Muntenia, Oltenia and Banat, but also in the western region of Crișana(3).

Even though both human and CanL cases stopped being reported after the malaria eradication program in 1958, it is clear that *L. infantum* life cycle can be completed in south of Romania under the right circumstances (14). More recent studies on this subject, report both human and canine imported cases but there are also some recent reports of autochthonous CanL, as well as one case of autochthonous human case and one *L. infantum* infection in a jackal(6, 7, 12, 13, 16).

The first recent autochthonous case of human leishmaniosis, reported in 2013, was described in a patient from the north of Romania, working in the southern county of Dolj, with no history of travel abroad(7). Similarly, the first case of *L. infantum* infection in a jackal was reported in 2017, also in the county of Dolj(13).

The first case of canine leishmaniosis and the first epidemiological study in dogs were reported in 2014 and 2016, respectively (6, 12). The dogs from both these reports originated from the southern county of Valcea, where the seroprevalence was found 3.7%, similar to the results of the present study(6, 12).

The most recent information about sandflies in Romania reports the presence of *P. neglectus* and *P. perfiliewi* in the South of the country, and of *P. perfiliewi* also in the East(1, 2). The presence of autochthonous *L. infantum* infections in dogs, humans and wildlife, as well as sandflies, known to be vectors of *L. infantum* shows that this infection is present in the south of Romania. More studies are needed to determine if these reports are just cases of local transmission from imported infected dogs or if CanL is becoming endemic in Romania.

Because the dog is the reservoir species for *L. infantum*, it is indispensable for the transmission of this parasite in a territory, and it is the best species to monitor in order to check for the presence of this infection. There is limited data about the prevalence of *L. infantum* in the south of Romania, in counties such as Dolj and Vâlcea and there is still a need for more studies in this region. There is no data about *L. infantum* infections in the south-east of Romania and it is necessary to investigate this region as well. In this study, we investigate the seroprevalence of *L. infantum* infections in the south-east of Romania and discuss the results, together with other relevant studies from this region, in order to better understand the possibility of

re-emergence of *L. infantum* infections, in Romania.

We report here that dogs from the South-East of Romania are indeed still exposed to *L. infantum*.

Future epidemiological studies on CanL should also use more specific methods such as qPCR. It is also important to continue monitoring *L. infantum* infections in humans and wildlife. Studies focused on the distribution of sandflies and their possible infection with *L. infantum* would also help identify regions where there is a higher risk of infection.

CONCLUSIONS

Canine leishmaniosis is one of the most important parasitic diseases of dogs from the Mediterranean area that can also have an impact on human health. Recent studies show that the distribution of this infection is changing and there is the possibility that it is re-emerging in Romania. In the present study, the seroprevalence of this infection in sheltered dogs from the South-East of Romania is reported. These are hopefully results that will contribute to the knowledge on the presence of *L. infantum* infections in Romania.

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