

Serological and entomological studies of canine leishmaniosis in Croatia

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ŽIVIČNJAK, T., F. MARTINKOVIĆ, C. KHOURY, G. BONGIORNO, S. BOSNIĆ, D. LUKAČEVIĆ, M. MAROLI: Serological and entomological studies of canine leishmaniosis in Croatia. Vet. arhiv 81, 99-110, 2011.

ABSTRACT

In Croatia, canine leishmaniosis has been recognized in the southern littoral area (Dalmatia) as a re-emerging disease since 1997. This paper reports the findings of a survey carried out 2.5 years after the suspension of control measures for canine leishmaniosis. Entomological and cross-sectional serological surveys were carried out in the Kaštela municipality in the Split-Dalmatia County (where stable foci of canine leishmaniosis were reported previously), and in a few localities from the Šibenik-Knin county where a few unstable foci were previously reported. Serological surveys detected a focus with 31% seropositive dogs in Rudine (the known enzootic area); a tendency for a new stable focus appearance in the Šibenik-Knin County (Rogoznica), with cumulative seropositivity of 13.5% in both areas. Entomological studies evidenced two competent *Leishmania* vectors, *Phlebotomus neglectus* and *P. tobbi*; the first was prevalent (75.9%) being also the species much more associated to habitats where dogs are present. Moreover, prevalence for *P. neglectus* females feeding on human blood reached 30%.

Key words: *Leishmania infantum*, monitoring, dog, sand fly, Croatia

Introduction

Canine leishmaniosis (CanL) caused by *Leishmania infantum* is a widely distributed and endemic zoonotic disease in the Mediterranean basin. Dogs are considered the main domestic reservoir of *L. infantum* for human infection (MORENO and ALVAR, 2002; ALVAR

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et al., 2004). Clinically ill dogs manifest different clinical signs and variable degrees of severity. In previous seroepidemiological surveys, a high proportion of seropositive dogs showed no clinical signs of leishmaniosis (SOLANO-GALLEGO et al., 2001; ŽIVIČNJAK, et al., 2005; DANTAS-TORRES et al., 2006). Infected dogs, even apparently healthy, are sources of infection for vectors (MOLINA et al., 1994). Female sand fly species belonging to *Phlebotomus* (Old World) or *Lutzomyia* genera (New World) are the proven vectors of *Leishmania* sp (KILLICK-KENDRICK, 1999; SHARMA and SINGH, 2008). Since 1997 CanL in Croatia has been proven parasitologically and serologically in hundreds of dogs (ŽIVIČNJAK et al., 1998 and 2007) and during 2003 cumulative seropositivity in the Split area reached 15% (ŽIVIČNJAK et al., 2005). All diseased and seropositive dogs visited or lived in central and southern parts of the region of Dalmatia. Due to the epizootiological pattern of leishmaniosis in Dalmatia, obligatory testing (free of charge for the owners) for all dogs exhibiting evident clinical symptoms of leishmaniosis (in the Šibenik-Knin, Split-Dalmatia and Dubrovnik-Neretva Counties) and medication with alopurinol for confirmed CanL cases was decreed by the Croatian Ministry of Agriculture, Forestry and Water Management from 2002 to 2005. During this period, efforts to serologically monitor the asymptomatic canine population in the enzootic area were also implemented. The advantage of the decreed measures was that veterinarians and dog owners in the known enzootic area became used to demanding “free blood tests” not only for clinically ill, but also for the other dogs in the cohort. Simultaneously, dog owners were encouraged to use dermal application of chemical compounds to protect dogs from sand fly bites. In 2005 obligatory testing of dogs in the enzootic area was perceived as too expensive, and it was ultimately suspended in 2006. The cumulative seropositivity of dogs from the whole enzootic area (three Dalmatian counties) recorded in 2005 was 7.9%, and monitoring in the Dubrovnik-Neretva County in 2006 recorded 8% seropositive dogs (ŽIVIČNJAK et al., 2007). Recent entomological surveys (BOSNIĆ et al., 2006) carried out in Dalmatia, revealed sand fly species of the *Phlebotomus* genus, among which three belonged to the *Larroussius* subgenus, being proven *L. infantum* vectors (*P. tobbi*, *P. neglectus* and *P. perfliewi*). Human visceral leishmaniosis has been sporadically reported in southern Croatia; it is not considered a major public health problem in Croatia (MULIĆ et al., 2002), with a mean annual incidence of 0.4/100.000 population (ANONYM., 2003). The aim of the survey was to estimate the circumstances for further spread of CanL, considering the vector abundance, suspension of the decreed measures and consequential canine monitoring abatement.

Materials and methods

Study area. The entomological and cross-sectional serological survey was carried out during the end of June 2008 in the known CanL enzootic area in the Split-Dalmatia County /Rudine, municipality Kaštela, situated 14 km westward from Split and 2 km inland from

the coastline/ where stable foci of CanL had been reported previously. Furthermore, in the adjacent Šibenik-Knin County a few localities came under the survey (i.e. Žaborić, Vadalj, Široke, Kruševo and Rogoznica). Although stable foci of CanL had been reported previously in the Šibenik-Knin County (only from the towns of Drniš and Knin; 35 and 55 km respectively away from surveyed area), CanL had never been reported in Žaborić, Vadalj, Široke and Kruševo. In the town of Rogoznica a single isolated case of CanL was reported originating from a cohort of nine dogs, 4 years ago. Žaborić and Rogoznica are close to the coastline; Vadalj, Široke and Kruševo are approximately 7 km from the coastline. The location and the characteristics of sites surveyed are presented in Fig.1 and Table 1.



Fig. 1. Study area (Localities in survey indicated by numbers: 1. Rudine; 2. Žaborić; 3. Vadalj; 4. Široke; 5. Kruševo; 6. Rogoznica). Dashed line is indicating border between Šibensko-Kninska (left) and Split-Dalmatija County (right).

Animals and blood sampling. The survey was carried out on 74 dogs (aged 10 months or more) regarded as healthy by their owners, originating from all the localities (referred to in Table 1), except Žaborić. All dogs were clinically examined for any signs of CanL, including lymphadenopathy, dermatitis, hair loss and ocular changes. In Rudine, blood sample collecting was organised at a veterinary clinic. 13 samples were taken during the field study, and a further 16 samples were sent by the local vet over the next few days

(a total of 29 sera). 45 dogs originated from the Šibenik-Knin County. Blood collecting from dogs in Vadalj, Široke and Kruševo was carried out in three kennels of local dog owners. In Rogoznica blood collection was organised at the local hunting lodge. The dogs' ages ranged from 10-144 months (average 45 months). In the surveyed group there were 40 females, and 34 males. Half of the group were hunting dogs (N=37), 18 were guard dogs and sheep dogs, 19 were bred or kept as pets. Blood samples were obtained with the owners' approval. Five mL of venous blood was collected from all 74 dogs by brachial vein puncture. Serum was separated, transported to the laboratory and kept at -20 °C until further use.

Serology. *In vitro* cultivation, antigen preparation and indirect immunofluorescence were performed as described by MANCIANTI et al. (1996), with modifications (MARTINKOVIĆ and MARINCULIĆ, 2006). A total of 74 canine sera samples were examined by indirect immunofluorescence test in serial dilutions (cut-off $\leq 40^{-1}$) with home antigen. The titre $\geq 80^{-1}$ was regarded as positive. Two positive sera samples (titre $\geq 1280^{-1}$ and titre $\geq 640^{-1}$) from clinically ill dogs with parasitologically proven diagnosis and two negative control sera (titre $\leq 40^{-1}$) from healthy six-month-old puppies from the enzootic region of Croatia (City of Split) were included in every assay. All sera were tested in duplicate and all were retested at least once.

Sand fly collections. Four collecting sites were selected at the dog owners' households, the same places where canine blood samples were collected (Rudine, Vadalj, Široke and Kruševo). CDC miniature light traps (Hausherr's Machine Works, Toms River, NJ, U.S.A.), castor oil sticky traps (20 × 20 cm) and battery-operated hand aspirators were used outside at peri-domestic sites, inside kennels and chicken houses. Hand aspirators were also used for collections inside bedrooms in Kruševo. In Žaborić, were surveyed 28 road wall holes with castor oil sticky traps left for two days. In Rogoznica no sand fly collections were carried out. CDC light traps were used for collecting adult sand flies during their nocturnal activity. In the CDC light traps, flies were kept alive in a net cage and therefore stored in absolute ethanol for further analyses. Battery-operated hand aspirators were used for collecting adult sand flies in their resting places in human and animal dwellings. Collected sand flies were immediately stored in absolute ethanol. Castor-oil sticky traps were examined a few hours after collecting, and the phlebotomines caught were collected with a paint brush and stored in absolute ethanol for further analyses.

Sand fly species identification. The phlebotomine species identification was performed by examining the morphology of the male genitalia, female spermatheca and pharynxes. Specimens were identified by their morphological characteristics, according to THEODOR (1958) and LÉGER et al. (1983).

Identification of blood meals. Before testing, blood-fed females were identified to species level by removing the head and the terminal segments of the abdomen containing the spermathecae. Blood meal origin was determined by direct ELISA on nitro-cellulose membrane (dot-ELISA) according to BONGIORNO et al. (2003) with commercial (Sigma Immunochemical Co) anti-sera IgG peroxidase conjugates (anti-human, anti-dog and anti-chicken).

Results

Canine serology results. In Rudine in the Split-Dalmatia County, 29 dogs were tested and 9 reacted positive. In the Šibenik-Knin county 45 dogs were tested; only one dog from Rogoznica reacted positive, and the others were seronegative. Cumulative results of canine serology are shown in Table 2. The seropositive dogs' origin, age, gender, race and titre are shown in Table 3.

Sand fly fauna composition. Cumulative results of sand fly collection are shown in Table 4. During the field survey, 353 sand flies belonging to the genera *Phlebotomus* and *Sergentomyia* were caught. Two competent *Leishmania* vectors were found, *P. neglectus* and *P. tobbi*, the first was the most abundant (75.9%) being also the species much more associated with habitats where dogs are present. *P. papatasi* (12.2%) were recorded in chicken houses, kennels and bedrooms. Outside settlements, inside the road wall holes, the only species recorded was *Sergentomyia minuta* (3.7%).

Blood meal identification. Blood meal identification was performed on 16 engorged *P. papatasi* females and 20 engorged *P. neglectus* females respectively. The results of blood meal identification are shown in Table 5.

Table 1. Characteristics of sites surveyed

N	County	Locality	Altitude Asl (m)	Latitude Longitude
1	Split-Dalmatia	Rudine	73	N 43° 33' 741'' E 16° 19' 220''
2	Šibenik-Knin	Žaborić	36	N 43° 40' 38'' E 15° 56' 99''
3	Šibenik-Knin	Vadalj	228	N 43° 34' 334'' E 15° 59' 309''
4	Šibenik-Knin	Široke	214	N 43° 35' 781'' E 16° 00' 833''
5	Šibenik-Knin	Kruševo	187	N 43° 35' 100'' E 16° 02' 604''
6	Šibenik-Knin	Rogoznica	1	N 43° 31' 29'' E 15° 58' 12''

Table 2. Cumulative results of canine serology

Locality	Seronegative dogs	Seropositive dogs	Total
Rudine	20 (69%)	9 (31%)	29
Vadalj	13 (100%)	-	13
Široke	3 (100%)	-	3
Kruševo	3 (100%)	-	3
Rogoznica	25 (96.2%)	1 (3.8%)	26
Overtotal (%)	64 (86.5)	10 (13.5)	74 (100)

Table 3. Origin, age, gender, race and IIF titre of seropositive dogs

Locality	Age in months	Gender	Race	Titre ⁻¹
Rudine	13	male	German Shepherd	2560 ⁻¹
Rudine	15	male	hunting dog	2560 ⁻¹
Rudine	48	female	Husky	80 ⁻¹
Rudine	60	male	mongrel	5120 ⁻¹
Rudine	96	female	mongrel	320 ⁻¹
Rudine	10	male	English Bulldog	320 ⁻¹
Rudine	10	female	French Bulldog	640 ⁻¹
Rudine	36	female	English Bulldog	2560 ⁻¹
Rudine	20	female	mongrel	160 ⁻¹
Rogoznica	40	female	hunting dog	320 ⁻¹

Table 4. Cumulative results on sand fly collections

Locality and method ^a	Collection sites	Specimens collected	Species			
			<i>P. neglectus</i> (%)	<i>P. tobbi</i> (%)	<i>P. papatasi</i> (%)	<i>S. minuta</i> (%)
Rudine (Kaštel Novi)						
ST	chicken pen, wall	12	-	-	-	12
CDC	chicken pen, kennel	10	7	1	2	-
HC	chicken pen, kennel	16	-	-	16	-
Total		38	7 (18.4)	1 (2.6)	18 (47.4)	12 (31.6)
Žaborić						
ST	Barbacane (28 holes)	1	-	-	-	1
Total		1				1 (100)
Vadalj						
CDC	kennel	74	71	3	-	-
HC	kennel	3	-	-	3	-
Total		77	71 (92.2)	3 (3.9)	3 (3.9)	
Široke						
ST	chicken pen	4	-	-	4	-
CDC	chicken pen, kennel	218	188	25	5	-
HC		13	2	-	11	-
Total		235	190 (80.9)	25 (10.6)	20 (8.5)	
Kruševo						
HC	bedroom	2	-	-	2	-
Total		2			2 (100)	
Overtotal		353	268 (75.9)	29 (8.2)	43 (12.2)	13 (3.7)

^a ST, sticky traps; CDC, light traps; HC, hand collections

Table 5. Results of blood meal identification

Species	Origin of sand fly blood meal				total
	human	dog	chicken	not identified	
<i>P. papatasi</i>	13	1	-	2	16
<i>P. neglectus</i>	6	5	3	6	20

Discussion

Although zoonotic visceral leishmaniosis is a re-emerging disease in the Mediterranean area, it has not been systematically monitored and documented (DUJARDIN, 2006; DUJARDIN et. al., 2008). It has been estimated that at least 2.5 million dogs are infected in South-western Europe (MORENO and ALVAR, 2002). Moreover, the disease is spreading northwards into the foothills of the Alps and the Pyrenees (MAROLI et al., 2008; FERROGLIO et al., 2005). The endemicity of CanL is associated with the distribution and abundance of vectors, consisting of several sand fly species of the subgenus *Larroussius* (KILLICK-KENDRICK, 1999). Information on the spread of the autochthonous CanL is essential in order to define effective control measures for zoonotic leishmaniosis. The control of both the canine and the human disease depends directly on effective control of CanL and sand fly control.

Since the decreed control measures in Dalmatia were suspended, it has been quite impossible to organize further widespread monitoring because of the lack of funds. Only some of the clinically diseased dogs are admitted to the local veterinarians and procedure with infected dogs is not regulated. Vets could recommend medication with follow-up or euthanasia, but nobody is responsible for control of the implementation. Briefly, dog owners make a decision whether to request clinical examination or not, also whether to treat the sick animal or not. Monitoring in the cohort where CanL is recorded should be paid for by the dog owner, and consequently nobody has requested it. It is relevant to note that hunters in Dalmatia usually own ten or even more hounds in a household; they swap, borrow, lease, sell and resell dogs and usually do not use any preventive measures against sand fly bites.

During the study, we identified a highly enzootic area in Rudine in the Split-Dalmatia County (with a cumulative seropositivity prevalence of over 31%). Three years ago in Rudine three dogs were detected with CanL in a kennel. One dog was euthanized, two were sold during alopurinol medication; serological monitoring has never been performed in the cohort. During the current survey, the same breeder had another three apparently healthy seropositive dogs, and six seropositive dogs were also detected in five households in Rudine. Serological monitoring in Rudine could detect infected dogs if it were performed on regular basis. In the adjacent Šibenik-Knin County, only one seropositive dog was detected in Rogoznica, but it should be mentioned that it originated from just the same household as the dog with CanL four years before. It was born after the infected dog had been euthanized. The explanation might be that at least one infected, apparently healthy dog had remained. During that period, the owner sold some dogs, he also “got rid of” some, but he also introduced new animals. We may anticipate that if the measures had not been suspended, monitoring in Rogoznica could have detected the infection earlier. Unfortunately, in Rogoznica we cannot organise an entomological survey, but

we may presume sand fly presence. The absence of seropositive dogs in Vadalj, Široke and Kruševo might be transient; we confirmed vectors presence and an abundance in the whole surveyed area and the *Leishmania* life cycle could begin with the introduction of infected dogs to the native cohort. Knowledge of the host preferences of phlebotomine sand flies under natural conditions is essential to understand their vectorial capacity in different leishmaniosis foci (ROSSI et al., 2008). The proportion of *P. neglectus* females fed on human blood (prevalence 30%) is a valid indicator of the epidemiologic importance of the vector control in the endemic/enzootic area. We may expect the spread of CanL and the sand fly vector towards the north and inland territories, as outbreaks are taking place in Italy (MAROLI et al., 2008; FERROGLIO et al., 2005; MOROSETTI et al., 2009). We should not only attribute the lack of money for the suspension of the control measures previously implemented in Croatia. Relevant health policies in other countries with indigenous CanL facilitated the decision. To reduce the transmission of *Leishmania* from dogs to vectors, besides vector control, it is necessary to diagnose canine leishmaniosis as early as possible. Serological diagnosis is considered essential for detecting the prevalence of infection (PAPADOPOULOU et al., 2005). Since we were only able to obtain samples from 74 dogs, out of approximately 5000 dogs living in the area, those samples should not be considered representative of the whole dog population in the area studied. Nevertheless, although the survey was conducted in a restricted area, these results give us enough information to expect a further rise in the incidence rate for both CanL and zoonotic leishmaniosis in the whole enzootic area, because the disease is generally neglected and is not under control at all. We may also anticipate the further spread of CanL due to the vectors present and their abundance in the surveyed area. Efforts should be made to prevent CanL in the known enzootic region(s); measures should be implemented as follows: monitoring on a regular basis (reservoirs and sand fly vector), vector control and finally, education of veterinarians and dog keepers. Further studies are needed to evaluate the spread of sand fly species belonging to the *Larroussius* subgenus, both in coastal and inland territories of littoral Croatia, to identify the areas at risk for the disease.

Acknowledgements

The investigations were financially supported by Croatian Ministry of Science, Education and Sports through scientific project *Spread of canine leishmaniosis and its sandfly vectors in the littoral Croatia* (053-0532266-224) and through Technical Assistance Information Exchange Instrument (TAIEX) Expert Mission on control of *L. infantum* vectors in Croatia (ref.code: AGR IND/EXP 30063) by European Commission Institution Building Unit.

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Received: 11 November 2009

Accepted: 29 January 2010

ŽIVIČNJAČ, T., F. MARTINKOVIĆ, C. KHOURY, G. BONGIORNO, S. BOSNIĆ, D. LUKAČEVIĆ, M. MAROLI: Serološka i entomološka istraživanja lišmanioze u pasa u Hrvatskoj. Vet. arhiv 81, 99-110, 2011.

SAŽETAK

Na jugu hrvatskoga priobalja (Dalmacija) lišmanioza pasa u ponovnom je porastu od 1997. godine. U ovom radu izneseni su rezultati istraživanja provedenih 2,5 godine nakon ukidanja obveznih mjera suzbijanja lišmanioze pasa. Serološko i entomološko istraživanje provedeno je na području općine Kaštela u Splitsko-dalmatinskoj županiji (područje s poznatim stalnim žarištima pasje lišmanioze) i na nekoliko lokacija Šibensko-kninske županije gdje je zabilježeno samo povremeno javljanje lišmanioze. Serološkim je istraživanjima otkriveno žarište s 31% serološki pozitivnih pasa u Rudinama (enzootsko područje) s tendencijom nastanka novoga stalnog žarišta u Šibensko-kninskoj županiji (Rogoznica), a ukupno je bilo 13,5% serološki pozitivnih pasa. Entomološkim istraživanjima dokazana je prisutnost dviju vrsta prijenosnika koji podržavaju razvoj roda *Leishmania*, *Phlebotomus neglectus* i *P. tobbi*; prvi je mnogo zastupljeniji (75,9%) i češće prisutan tamo gdje ima pasa, a čak 30% pretraženih ženki *P. neglectus* hranilo se na ljudima.

Ključne riječi: *Leishmania infantum*, monitoring, pas, papatač, Hrvatska
