Canine leishmaniasis surveillance program in a San Marino Republic kennel

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Republic of San Marino,
Surveillance.

Summary
The Republic of San Marino is an autonomous State that, in view of its geographical and environmental features, can be considered a part of the Northern Italian territory, where the canine leishmaniasis (CanL) is endemic. In the past, a CanL focus in the Republic's kennel was described. As a consequence of this epidemiological situation, a surveillance program was carried-out covering a 6-year period (2006-2012). A total of 1,094 sera were collected from 420 kennel dogs and examined for antibodies to *Leishmania infantum* by the indirect fluorescent antibody test (IFAT). Eighty-eight (21%) dogs resulted IFAT positive (antibody titre ≥1/40). The overall seroprevalence increased in the first 4 years (2006-2010), going from 5.5% to 26.8% and then decreased in the 2 following years going to 17.9% (2011) and 3.9% (2012). The cumulative incidence constantly increased from 0.6% to 2.6%. This trend could be attributed to a changed infection pressure due to the dog turnover in the kennels. According to the observed incidence values, the CanL focus seems to be stable, supported by autochthonous transmission, new case introduction and *Leishmania* spp. circulation in owned dogs in the same area.

Programma di sorveglianza della leishmaniosi canina in un canile della Repubblica di San Marino

Parole chiave
Cane,
Canile,
Immunofluorescenza indiretta,
IFI,
Leishmania infantum,
Repubblica di San Marino,
Sorveglianza.

Riassunto
La Repubblica di San Marino è uno Stato autonomo confinante con l'Emilia Romagna, dove la leishmaniosi canina (LCan) è endemica. Nel presente studio sono descritti i risultati di un programma di sorveglianza della durata di 6 anni attivato nel canile della Repubblica di San Marino, a seguito di un precedente focolaio di LCan all'interno di tale struttura. Complessivamente sono stati analizzati, mediante immunofluorescenza indiretta (IFI), 1.094 sieri prelevati da 420 cani, 88 dei quali sono risultati positivi (IFI ≥ 1/40). Nel periodo 2006-2010 la sieroprevalenza ha presentato un incremento dal 5,5% al 26,8%, mentre negli anni 2011 e 2012 è stata registrata una diminuzione al 17,9% e 3,9%, rispettivamente. L'incidenza cumulativa è aumentata costantemente da 0,6% a 2,6%. Tale andamento può essere imputato a modifiche della pressione di infezione conseguenti al turnover degli animali. Sulla base dei dati di incidenza osservati, il focolaio di LCan nel canile della Repubblica di San Marino sembra essere attualmente stabile, supportato dalla trasmissione autoctona dell'infezione, dall'introduzione di nuovi casi e dalla circolazione di *Leishmania* spp. in cani di proprietà presenti nella stessa area geografica.

Introduction

Canine leishmaniasis (CanL) due to *Leishmania infantum* is endemic in the whole Mediterranean basin, representing an important public health problem, since dog is considered the main reservoir of the infection (13).

In Italy, until the 1970s, CanL was endemic in Central and Southern regions, including islands, while Northern Italy was considered free from CanL, with the exception of Liguria and few areas in the Emilia-Romagna region. Since the mid-1980s, a progressive spread of the infection has been observed both in the old foci of Central and Southern regions and in the previously free Northern ones (8, 10, 11, 19, 20, 26, 28, 32, 35).

In the Emilia-Romagna region, in the early 1970s, a severe human visceral leishmaniasis outbreak occurred without evidence of a canine reservoir, although seropositive dogs were identified (18, 24). Several years later, new foci of CanL and the re-emergence of the old one have been reported (3, 20, 22). Starting with 2007, the Emilia-Romagna region promoted a regional surveillance program focusing on vector-borne diseases, including leishmaniasis (34).

The Republic of San Marino is a small autonomous State within the Italian territory, neighbouring CanL endemic areas of the Emilia-Romagna region (3, 6). In 2003-2004, a CanL autochthonous focus in the Republic kennel was observed, showing seroprevalence and incidence values of 7.1% and 6.6%, respectively (4). Further investigations allowed for isolating a strain of *L. infantum* zymodeme MON-1. Moreover, *Phlebotomus perfiliewi*, the proved *L. infantum* vector and typical sand fly in the Emilia-Romagna foci (2, 3, 15), was detected in the kennel. In the present study we report the results of a 6-year surveillance program carried out to evaluate the dynamics of the infection in the Republic's kennel.

Materials and methods

The Republic of San Marino (43° 46' N, 12° 25' E) is located in the middle of Italy and borders with Rimini and Pesaro-Urbino provinces, respectively in the Emilia-Romagna and Marche regions (Figure 1). The land covers an area of 61,196 km^2, mostly hilly and clayey. The climate is continental, with hot summers and cold winters and heavy snowfalls. The summer mean temperatures range between 20°C and 30°C, with peaks of 35°C, the winter temperatures range between -5°C and 10°C. The kennel is sited in a hilly area used for the intensive cultivations of wheat, grapevine and orchard, surrounded by rich vegetation.

Figure 1. *Localization of the Republic of San Marino within the Italian territory.*

Both roaming or abandoned dogs are sheltered in the kennel: the animals are either re-housed or remain there permanently (31). The canine population consists of about 160 dogs. A mean of 38 dogs enters into the kennel each year.

The dog's health conditions are checked by the public Veterinary Service of the Republic of San Marino. After the first detection of the CanL focus, each year, from June to September, all dogs are provided with deltamethrin-impregned collars against sand fly bites. Once a year, before the transmission season, serological tests are performed on all housed animals as well as on the new entry dogs.

From 2006 to 2012, a total of 1094 sera from 420 kennel dogs were collected and analysed (Table I). The number of animals tested every year changed, according to the turn over of the animals in the kennel. Only 40 dogs, which never moved from the kennel, were examined throughout the study period.

Anamnestic data (sex, estimated age, breed, origin, date of entry in the kennel, clinical signs and therapeutic treatment referred to leishmaniasis) were recorded for each dog. Two hundred and fifty six out of 420 (61%) animals were male and 159 (37.9%) were female, while sex data were not available about 5 dogs (1.1%). Two hundred and twenty two out of 420 (52.9%) dogs were crossbreed dogs, 70 (16.7%) hunting dogs, 37 (8.8%) belonged to other breeds, 91 (21.7%) were breed-undetermined. Referring to the age, 194 out of 420 (46.2%) were young (< 3 years old), 76 (18.1%) adult (3-7 years old), 57 (13.6%) old (≥ 7 years old), while the age was not established for 93 (22.1%) animals. One hundred and forty two out of 420 dogs originated from the Republic of San

Marino and 5 from Emilia-Romagna and Abruzzo regions; the geographical origin was not reported for 254 dogs. Clinical signs of leishmaniasis were observed in 21 dogs in at least one control.

The samples were tested by the indirect fluorescent antibody test (IFAT), the 'gold standard' test for the diagnosis of CanL, according to the laboratory procedures described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (36). An in-house antigen consisting of promastigotes of *L. infantum* strain MHOM/TN/80/IPT1 was used. The cut-off was set at 1/40. Animals with IFAT titre of 1/40 and 1/80 were considered exposed to the parasite. Dogs showing antibody titres ≥ 1/160 were considered infected and treated (14) by 100 mg/kg/SID (*semel in die*; once a day) s.c. for 28 days in association with 10 mg/kg/BID (*bis in die*, twice a day) allopurinol *per os* for 6 months; then meglumine antimoniate was substituted with 2 mg/kg/SID miltefosine *per os* for 28 days (33), because of its easier administration.

Data were collected into Microsoft Excel®, and then analysed by Statistical Package for Social Science version 14 (SPSS, Inc., Chicago, USA). The seroprevalence (all seropositive dogs per year/ dogs sheltered per year) and the cumulative incidence (new cases with IFAT titre ≥ 1/160 per year/ new cases with IFAT titre ≥ 1/160 per year + population time at risk) were calculated from 2006 to 2012 and from 2007 to 2011, respectively.

The chi-square test was used to evaluate the relationships between IFAT results and the others variables and to compare the incidence value with the number of new entries per year ($p \leq 0.05$). The prevalence values per year were compared using the Fisher's exact test ($p \leq 0.05$).

Table I. *Dogs tested for canine leishmaniasis from 2006 to 2012 in San Marino Republic.*

Year	Total dogs	New Entry
2006	165	28
2007	176	51
2008	173	55
2009	166	39
2010	153	45
2011	134	26
2012	127	28

Results

Serological results are reported in Table II and III.

Out of 420 dogs tested, 88 (21%) were IFAT positive (≥ 1/40) in at least 1 test during the entire surveillance period. Fourteen dogs showed an active infection (IFAT titres ≥1/160): 5 of them were tested only once and no information about therapy has been reported. The remaining 9 dogs were treated and after the therapy, 6 showed IFAT titres fluctuating from doubtful to negative, 2 dogs confirmed the previous IFAT titres and one dog was tested and treated only once.

Table II. *Serological results on canine leshmaniasis from 2006 to 2012 in San Marino Republic.*

		Year						
		2006	2007	2008	2009	2010	2011	2012
IFAT results	<1/40	156	159	151	141	112	110	106
	1/40-1/80	6	13	21	23	37	18	4
	≥1/160	3	4	1	2	4	6	1

IFAT = indirect fluorescent antibody test

Table III. *Serological results on canine leshmaniasis from 2006 to 2012 divided by resident and new entry dogs in a kennel of San Marino Republic.*

Year	Resident dogs			New entry dogs		
	<1/40	1/40-1/80	≥1/160	<1/40	1/40-1/80	≥1/160
2006	129	5	3	27	1	/
2007	117	6	2	42	7	2
2008	106	12	/	45	9	1
2009	108	18	1	33	5	1
2010	78	29	1	34	8	3
2011	88	15	5	22	3	1
2012	95	3	1	27	1	/

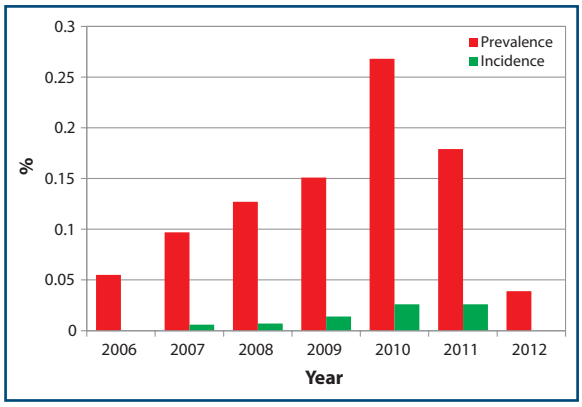

Figure 2. *Prevalence of canine leshmaniasis in dogs of San Marino Republic from 2006 to 2012; incidence from 2007 to 2011.*

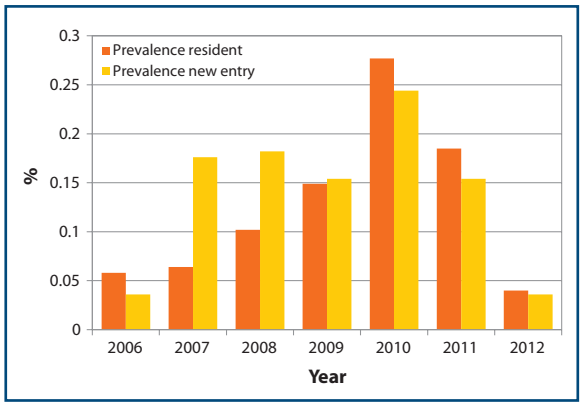

Figure 3. *Prevalence of canine leshmaniasis calculated on resident and new entry dogs in a kennel of San Marino Republic from 2006 to 2012.*

The overall seroprevalence increased from 5.5% (2006) to 26.8% (2010) and then decreased to 17.9% (2011) and 3.9% (2012) (Figure 2). Seroprevalence calculated on resident dogs modified significantly ($p \leq 0.05$), increasing from 5.8% (2006) to 27.7% (2010) and then decreasing to 4.0% (2012) (Figure 3). The cumulative incidence value constantly increased from 0.6% (2007) to 2.6% (2011) (Figure 2). It is noteworthy that no new cases have been recorded in 2012.

Forty out of 420 dogs have been controlled throughout the monitoring period, 27 of them proved constantly seronegative, while 11 animals fluctuated from a negative to exposed status and 2 seroconverted to IFAT titres ≥1/160 and were treated.

In frequency order, lymphadenopathy, dermatitis, onychogryphosis, alopecia, and poor condition were observed. The comparison of the overall seroprevalence with sex, age, breed and clinical signs of the animals did not show a statistically significant difference ($p > 0.05$), as well as the comparison of the incidence value with the number of new entries. With reference exclusively to 2009-2011 period, the rate of the old (≥7 years old) seropositive dogs with clinical signs increased significantly from 4.2% (2009) to 6.7% (2011) ($p \leq 0.05$).

Discussion

In the first year of the surveillance program, a seroprevalence value (5.5%) lower than the one (7.1%) previously reported in the same focus (4) was observed. The decreasing of the value could be attributed to the euthanasia applied in 2004 to all seropositive dogs and to the antivectorial measures provided for those seronegative.

During the survey the seroprevalence and the cumulative incidence increased from 5.5% (2006) to 26.8% (2010) and from 0.6% (2007) to 2.6% (2010), respectively; then the seroprevalence decreased to 17.9% (2011) and 3.9% (2012), while the incidence value remained constant. This trend might be explained by the turnover of the animals in the structure, which could contribute to increase/decrease the infection pressure. A higher number of dogs entered the kennel from 2007 to 2010 than it did in 2011-2012 period. It is noteworthy that the greatest number of seropositive animals was introduced in 2010, when the highest seroprevalence was reported. Nevertheless, no significant association has been showed in comparing the prevalence of new entry and resident dogs. These results are different from those observed in a 'close system' in which no new dogs were admitted: a significant decreasing of seroprevalence and incidence was reported from the beginning to the end of the study period, related to the therapeutic and prophylactic measures applied (27).

Forty dogs permanently housed in the kennel have been monitored throughout the surveillance period, Although more than the half of them resulted constantly negative, 11 showed fluctuating antibody titres and 2 seroconverted to IFAT titres ≥1/160, suggesting a poor efficacy of the antivectorial measures.

Nine out of 14 dogs showing IFAT titres ≥ 1/160 in at least one control were subjected to recommended therapeutic protocols (14, 34). In most of the dogs, IFAT titres decreased confirming that an appropriate therapy can reduce the parasitic load. Although treated animals continue to harbour the parasite, the therapy represents a key factor to control the infection spread (14, 21), by inducing a significant reduction of the infectious load to sand flies.

No significant association between serological results and sex, breed and clinical signs was detected. Similar results have been reported in studies carried out in Spain and Greece (1, 12, 16), while significant

differences with respect to the sex and the breed have been observed in Croatia (37).

Starting with 2009 until 2011, an increased frequency of infected symptomatic old dogs was observed, showing how the risk of exposure to infection raises with the age of the animal, which also prompts a decreasing in the immune competence of the host (7, 25, 29).

Conclusions

According to the incidence values observed in the present study, the CanL focus in the Republic kennel seems to be stable, supported by autochthonous transmission, new case introduction and *Leishmania* spp. circulation in owned dogs in the same area (data not shown).

Incidence values reported are not very high if compared with those registered in a previous study conducted in another kennel (5) for a shorter period, which was probably insufficient to observe improvements related to control measures.

In the present study, the CanL prevalence is quite similar to the one reported in the kennels of the Emilia-Romagna region (17), mainly in the bordering Rimini province (9), an historical endemic area for about 10 years (2, 3, 6).

In general, the kennel is not a good epidemiological observatory, because the dog population is characterized by a turnover of free roaming or abandoned animals and data about the geographical origin often lack or are incomplete and incorrect. This was the case of the canine population described in this article, which was dynamic, variable and not foreseeable. Nevertheless, several data about the CanL spread in Italy are collected in kennels (5, 9, 23, 29, 30). Despite the limits related to the kennel features, the control of these facilities provides a useful tool to avoid the spread of the infection to free areas, which could easily escalate given the increased number online kennel dog adoptions.

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