Molecular evidence of Leishmania infantum *in* Ixodes ricinus *ticks from dogs and cats, in Italy*

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Summary
Leishmaniosis, caused by *Leishmania infantum*, is an endemic zoonosis in the Mediterranean basin. To date, phlebotomine sand flies are the only accepted biological vectors of *Leishmania* parasites to dogs and humans. The absence of the primary vector in autochthonous *Leishmania* outbreaks suggests a possible role of fleas or ticks as alternative vectors. In this study, 119 ticks were collected between August 2007-June 2008 and between March 2010-October 2010 from various animal species and humans living in Italian areas where canine leishmaniosis is endemic (*i.e.* rural areas of the North) and were tested for the presence of *L. infantum* DNA. Nine (7.5%) out of 119 ticks resulted PCR positive. All ticks were morphologically identified as *Ixodes ricinus* ticks, 3 from 1 cat, 6 from 4 dogs. To our knowledge, this is the first evidence of *L. infantum* DNA in ticks from cat, suggesting that the debate about the epidemiological role of ticks in canine leishmaniosis might be extended to feline leishmaniosis.

Analisi molecolare di Leishmania infantum *in zecche* Ixodes ricinus *da cani e gatti in Italia*

Parole chiave
Cane,
Gatto,
Ixodes ricinus,
Leishmania infantum,
Polymerase chain reaction (PCR),
Zecche.

Riassunto
La leishmaniosi sostenuta da *Leishmania infantum* è una zoonosi endemica nel bacino del Mediterraneo. Allo stato attuale delle conoscenze i flebotomi sono gli unici vettori riconosciuti per la trasmissione del microrganismo a cane e uomo. Tuttavia, il mancato rilevamento del vettore all'interno di focolai autoctoni di infezione ha indotto ad indagare sul possibile coinvolgimento di zecche o pulci come vettori alternativi. In questo studio, 119 zecche sono state prelevate in Italia settentrionale e centrale, tra agosto-giugno 2008 e marzo-ottobre 2010, in diverse specie animali e uomo. Le zecche sono state successivamente testate per la presenza di DNA di *Leishmania infantum*. Nove zecche (7,5%), risultate positive a PCR, sono state identificate morfologicamente come *Ixodes ricinus*. Tali parassiti sono risultati provenire da animali (gatto e cane) presenti in aree endemiche per leishmaniosi. Lo studio sembra essere la prima segnalazione di DNA di *Leishmania infantum* da zecche rimosse da gatto. Tale riscontro sembra suggerire che il dibattito sul ruolo delle zecche nell'epidemiologia della leishmaniosi nel cane possa essere esteso anche a quella del gatto.

Leishmaniosis, caused by *Leishmania infantum*, is an endemic zoonosis in the Mediterranean basin. Dogs are the primary domestic reservoir hosts; however in areas where visceral leishmaniosis is endemic, other, both domestic and wild, animal species have been found infected (Gramiccia 2011, Molina *et al.* 2012, Antoniou *et al.* 2013, Pennisi *et al.* 2013).

To date, phlebotomine sand flies (*Diptera: Psychodidae*) are the only accepted biological vectors of *Leishmania* parasites to dogs and humans, in nature (Killick-Kendrick 1999).

However, in recent years, autochthonous cases of canine leishmaniosis have been reported in areas where the presence of the primary vector has not been determined (Dantas-Torres *et al.* 2005). This finding posed the need to explore alternative ways of parasite transmission, suggesting also a possible role of fleas or ticks as alternative *L. infantum* vectors (Coutinho *et al.* 2005, Coutinho and Linardi 2007, Ferreira *et al.*2009, Silva de Morais *et al.* 2013).

In this study, ticks were collected from various animal species and humans living in Italian areas where canine leishmaniosis is endemic; the ticks were tested for the presence of *L. infantum* DNA.

One hundred and nineteen ticks were collected in 2 periods (August 2007-June 2008 and March 2010-October 2010) in 14 rural areas of the Northern and Central Italy, for purposes other than *L. infantum* investigation. Eighty-four ticks were removed from 14 dogs, 28 from 6 horses, 3 from 1 cat, 3 from 2 humans and 1 from a bovine.

Ticks were placed in vials containing 70% ethanol and morphologically identified using proper taxonomic keys (Halos *et al.* 2004, Mancianti *et al.* 2004, Varani *et al.* 2013). After identification, DNA was extracted from individual ticks using a commercial kit according to Aureli *et al.* (2012)

The efficiency of tick DNA extraction was evaluated by amplification of the tick mitochondrial 16S rRNA, according to Halos *et al.* (2004).

A conventional PCR amplifying a kinetoplast fragment of 145 bp was performed, using primers RV1 (5'-CTTTTCTGGTCCCGCGGGTAGG-3') and RV2 (5'-CCACCTGGCCTATTTTACACCA-3'), according to Lachaud and colleagues (Lachaud *et al.* 2002).

The reaction was conducted in a 50 µl final volume containing 50 mM KCl, 1,5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 µM of each deoxynucleoside triphosphate, 50 pmol of each primer, 1.25 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 5 µl of the DNA sample. The conditions for the polymerase chain reaction (PCR) were as follows: initial denaturation at 94°C (4 min), 40 cycles each consisting of denaturation at 94°C (1 min), annealing at 58°C (1 min), extension at 72°C (1 min). A final elongation step of 10 minutes at 72°C completed the reaction. Both the DNA from a *L. infantum* reference strain (MHOM/TN/80/IPT1) and a sample without *L. infantum* DNA were included in each PCR reaction as positive and negative control, respectively. PCR products were visualized on a 2% agarose gel in 1× TAE buffer with ethidium bromide staining. PCR products were purified using a commercial kit (QIAquick PCR purification kit, Roche, Basel, Switzerland) and sequenced in both directions (Bio-Fab Research, Rome, Italy). Sequences were compared with those available in GenBank database using NCBI BLAST[1].

Morphological identification as well as molecular results are shown in Table I.

Tick mitochondrial 16S rRNA gene was detected in all the samples. Nine (7.5%) out of 119 ticks tested positive to the PCR, showing 95-98% sequence similarity with *L. infantum* kDNA sequences available in GenBank database (accession numbers: EU370899.1; EU370893.1; Z35292.1; HQ585883.1; EU370895.1).

All the 9 ticks were morphologically identified as *Ixodes ricinus* ticks: 6 were adult engorged females, 3 adult males.

Three out of the 9 ticks were removed from a cat living in Montecatone area (Bologna province, Italy), 4 from 2 dogs living in the Gessi Bolognesi e Calanchi dell'Abbadessa Regional Park (Bologna Province, Italy) and 2 from 2 dogs in Forlì area (Forlì-Cesena province, Italy).

With regard to the animals from which PCR positive ticks were removed, no anamnestic data were available about dogs, while the anamnesis of the cat reported that the animal was apparently healthy.

The results of this study show the *Leishmania* kDNA presence in ticks removed from 4 dogs and 1 cat living in areas where canine leishmaniosis is endemic. Montecatone is near to territories of the Bologna province, where the infection has been reported both in humans and dogs (Pampiglione *et al.* 1974, Mollicone *et al.* 2003, Varani *et al.* 2013). It is noteworthy that Gessi Bolognesi-Calanchi dell'Abbadessa Regional Park is located in a territory bordering a stable focus of canine leishmaniosis in another area of Bologna province (Mollicone *et al.* 2003); in the Forlì province, autochthonous cases in dogs have also been reported (Baldelli, personal communication).

The finding of *Leishmania* DNA in ticks from dogs is not surprising, according to previous studies showing *L. infantum* DNA in *Ripichephalus sanguineus* ticks removed from *Leishmania*

[1] htpp://blast.ncbi.nlm.nih.gov/Blast.cgi.

Table I. *Sources and molecular results to* Leishmania infantum *in ticks collected in Northen and Central Italy between August 2007-June 2008 and between March 2010-October 2010.*

Localization of samples sites	Geographical coordinates	Tick sources	N. ticks/ Animal sources	Ticks species	Developmental stage and sex of ticks				Molecular results (n)
					Female	Engorged female	Male	Nymph	
Bologna Province									
Montecatone	44°21′12″N 11°42′5″E	cat	3	*I. ricinus* [a]		3			positive (3)
		dog	5	*R. sanguineus* [b]	2		3		negative
			1	*R. bursa* [c]	1				negative
		horse	3	*R. turanicus* [d]	1		2		negative
			1	*H. detritum scupense* [e]			1		negative
		horse	9	*R. turanicus*	2	3	4		negative
			1	*H. detritum detritum* [f]			1		negative
		horse	1	*H. marginatum marginatum* [g]	1				negative
			7	*R. turanicus*	3		4		negative
Gessi Bolognesi e Calanchi dell'Abbadessa Park	44°26′32″N 11°26′30″E	dog	1	*I. ricinus*		1			positive
		dog	17	*I. ricinus*	1	5	11		positive (3)
Castel de Britti	44°28′0″N 11°24′0″E	horse	2	*H. marginatum marginatum*			2		negative
Pianoro	44°23′0″N 11°20′0″E	horse	1	*I. ricinus*		1			negative
Ozzano dell'Emilia	44°27′0″N, 11°29′0″E	human	2	*R. turanicus*			1		negative
				I. ricinus			1		negative
		dog	1	*I. ricinus*		1			negative
San Giovanni in Persiceto	44°38′27″N 11°11′6″E	dog	3	*I. ricinus*	1			2	negative
		dog	14	*R. sanguineus*	5		4	5	negative
		dog	19	*R. sanguineus*	10		9		negative
Forlì-Cesena Province									
Forlì	44°13′21″N 12°2′27″E	dog	2	*I. ricinus*		1	1		positive (1)
		dog	2	*R. sanguineus*			2		negative
		dog	2	*I. ricinus*		2			positive (1)
Mercato Saraceno	43°57′0″N 12°12′0″E	horse	3	*I. ricinus*		3			negative
Modena Province									
Polinago	44°21′0″N 10°43′0″E	human	1	*I. ricinus*	1				negative
Ravenna Province									
Casola Valsenio	44°13′0″N 11°37′0″E	bovine	1	*R. bursa*			1		negative
Lido di Savio	44°18′10″N 12°20′45″E	dog	1	*R. turanicus*			1		negative
Lugo	44°25′0″N 11°55′0″E	dog	6	*R. sanguineus*	5		1		negative
Pesaro-Urbino Province									
Montecchio	43°49′37″N 12°48′8″E	dog	1	*I. acuminatus* [h]	1				negative
Republic of San Marino									
Republic of San Marino	43°46′N 12°25′E	dog	9	*R. sanguineus*	1				negative
				I. ricinus	8				negative

(a) = *Ixodes ricinus*; (b) = *Rhipicephalus sanguineus*; (c) = *Rhipicephalus bursa*; (d) = *Rhipicephalus turanicus*; (e) = *Hyalomma detritum scupense*; (f) = *Hyalomma detritum detritum*; (g) = *Hyalomma marginatum marginatum*; (h) = *Ixodes acuminatus*.

seropositive and seronegative dogs (Coutinho *et al.* 2005, Dantas-Torres *et al.* 2010a, Fontes Paz *et al.* 2010, Solano-Gallego *et al.* 2012), as well as in *Ixodes ricinus* ticks from a *Leishmania* naturally infected dog living in Central Italy (Trotta *et al.* 2012).

However to our knowledge, this is the first evidence of *L. infantum* DNA in ticks from cats.

No anamnestic data were available about the PCR positive cat, except the lack of clinical signs. This data do not exclude *Leishmania* infection, since feline leishmaniosis is often associated to mild clinical signs, limited to the inoculation site (Mancianti *et al.* 2004). In this respect, a previous study showed no statistical association between positive *Leishmania* PCR results and clinical status of cats (Sherry *et al.* 2011).

The *in vitro* model proposed by Prima *et al.* (2007) showed that *Leishmania* kDNA is rapidly degraded following amastigote death, thus its detection might suggest the viability of the parasite. Nevertheless, as highlighted by other authors (Otranto and Dantas-Torres 2010, Dantas-Torres *et al.* 2010b), the retrieval of *L. infantum* DNA in ticks does not confirm their vector competence.

Feline leishmaniosis has been described in several countries, mostly in areas where the infection is endemic, such as Spain, Italy, France, Portugal, Brazil (Dunan *et al.* 1989, Costa Durao *et al.* 1994, Pennisi *et al.* 2004, Martin-Sanchez *et al.* 2007, Magno da Silva *et al.* 2010).

The epidemiological role of the cat in *Leishmania* maintenance and spread has been long discussed, suggesting that cat may represent a secondary host reservoir rather than an accidental host (Gramiccia 2011). The evidence of the parasite transmission from naturally infected cats to phlebotomine vectors in Italy (Maroli *et al.* 2007) and in Brazil (Magno da Silva *et al.* 2010) suggested a sand fly-transmission of feline leishmaniosis.

Our results seem to indicate that the debate about the epidemiological role of ticks in canine leishmaniosis might be extended to feline leishmaniosis. To our knowledge, this is the first report of *L. infantum* DNA in ticks removed from cats. Nevertheless, the present study presents some strong limits, first of all that the ticks were collected for purposes other than *Leishmania* investigation, so concurrent serological or molecular analysis for *L. infantum* from the animals parasitized were not performed. Moreover, the animals previously tested were untraced to perform additional tests. Taking in account these considerations, the results of the present study should be considered preliminary to further and more focused investigations including a larger number of ectoparasitized cats, with the aim to assess the prevalence of *L. infantum* in ticks from cats and the possible correlation of pathogens detected in vectors and status of infection in animals.

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