Pathogens and symbionts in ticks: a survey on tick species distribution and presence of tick-transmitted micro-organisms in Sardinia, Italy

Giuseppe Satta, Valentina Chisu, Pierangela Cabras, Francesco Fois and Giovanna Masala

Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy

Correspondence
Giuseppe Satta
giuseppe.satta@izs-sardegna.it

Received 20 April 2010
Accepted 29 September 2010

A total of 1485 adult ticks were collected from mammalian hosts in south-eastern Sardinia, Italy, during the years 2007–2008. Ticks were identified and tested by PCR analysis for presence of *Rickettsia* species of the spotted fever group, *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Bartonella* species and *Leishmania* species. Among all tick species examined (*Rhipicephalus sanguineus*, *Rhipicephalus turanicus*, *Rhipicephalus bursa*, *Rhipicephalus pusillus*, *Hyalomma marginatum marginatum*, *Haemaphysalis sulcata* and *Dermacentor marginatus*), only *Hyalomma marginatum marginatum* produced negative results. A total of 22 pools belonging to the three tick species *Rhipicephalus sanguineus* (0.9 %), *Rhipicephalus turanicus* (4.5 %) and *Rhipicephalus pusillus* (100 %) were positive for *Rickettsia* species, while a total of five pools belonging to *Rhipicephalus sanguineus* (0.09 %), *Haemaphysalis sulcata* (16.7 %) and *D. marginatus* (7.8 %) were positive for *E. canis.* Five pools of *Rhipicephalus turanicus* (1.8 %) were positive for *A. phagocytophilum.* Positivity for *C. burnetii* was found in seven pools belonging to three tick species: *Rhipicephalus sanguineus* (0.5 %), *Rhipicephalus turanicus* (0.3 %) and *Haemaphysalis sulcata* (4.4 %). Finally, four pools belonging to *Rhipicephalus sanguineus* (0.09 %), *Rhipicephalus turanicus* (0.7 %) and *Rhipicephalus bursa* (1.1 %) were positive for *Bartonella* species. *Leishmania* species DNA was not detected in any of the tick pools examined. Data presented here increase our knowledge on tick-borne diseases in Sardinia, and provide a useful contribution to understanding their epidemiology.

INTRODUCTION

Tick-borne diseases are an emerging medical and veterinary problem. Ticks are implicated in the transmission of different pathogens such as viruses, bacteria, protozoa and filarial nematodes (Dantas-Torres, 2008). In the last few decades, there has been an increasing interest in zoonotic tick-borne diseases, which are considered as one of the most important zoonoses in Europe (Parola & Raoult, 2001). The family Ixodidae includes several relevant species, most of which belong to the genera *Haemaphysalis*, *Rhipicephalus*, *Dermacentor*, *Amblyomma* (Dantas-Torres, 2008) and *Hyalomma* (Black & Piesman, 1994). *Rhipicephalus bursa* and *Hyalomma marginatum marginatum* occur in all bioclimatic zones, while *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* occur essentially in the mesomediterranean bioclimatic zone. *Dermacentor marginatus* and *Haemaphysalis sulcata* are found frequently in the biotopes of the attenuated mesomediterranean and submediterranean bioclimates (Papadopoulos *et al.*, 1996). Ixodid ticks can transfer members of the spotted fever group (SFG) rickettsiae to vertebrates via salivary secretions, and among themselves both transtadially and transovarially (Beninati *et al.*, 2002). Canine monocytic ehrlichiosis is a cosmopolitan tick-borne disease of dogs that is primarily caused by *Ehrlichia canis* (Stich *et al.*, 2002). Recently, *Anaplasma phagocytophilum* causing disease in humans and animals was also found in *Ixodes* ticks (Foley *et al.*, 2008). *Coxiella burnetii*, the causative agent of Q fever 'worldwide zoonosis', has been detected in several tick species, but the role of ticks in transmitting the pathogen to humans is probably minimal (Psaroulaki *et al.*, 2006). The potential role of ticks as vectors of *Bartonella* species has been only hypothesized (Angelakis *et al.*, 2010). *Ixodes ricinus* ticks are competent vectors for *Bartonella henselae*, but further investigations are needed in order to evaluate their ability to transmit this pathogen (Cotté *et al.*, 2008). *Rhipicephalus sanguineus* has been demonstrated to be susceptible to infection by *Leishmania*, and to be able to mediate its transmission to experimental hosts (Coutinho *et al.*, 2005). *Rhipicephalus sanguineus* is considered a globalized tick and is able to transmit pathogens such as *Rickettsia rickettsii* (Dantas-Torres, 2008), *Ehrlichia* species and *Anaplasma* species (Sarih *et al.*, 2005), *C. burnetii*

Abbreviation: SFG, spotted fever group.

(Bernasconi *et al.*, 2002) and *Leishmania infantum* (Coutinho *et al.*, 2005).

METHODS

Study area. Sardinia is the second largest island in the Mediterranean Sea, with an area of 23 821 km^2. Ogliastra, one of the two collection areas, is a region of great naturalistic importance located in south-eastern Sardinia. On the northern side, Ogliastra is surrounded by the Gennargentu mountains, forests and green valleys covered by the typical Mediterranean maquis with *Cistus*, lentisk, myrtle and rosemary shrubs. The landscape is also characterized by cultivated coastal plains, watercourses and rocky sheer coasts. Many areas are dedicated to rearing and grazing of sheep, goats, bovines, swine and horses. Ogliastra is also an extraordinary habitat for wild animals such as mouflons, Sardinian deer, wild pigs and foxes and many birds. The second collection area, in the province of Cagliari, is located in southern Sardinia and is characterized by a wide diversity in geology, vegetation and landscape features, marked by the presence of mountains, great woods and Mediterranean maquis where rare animals such as wild cats, deer and wild pigs and several rare birds live. The rest is dedicated to rearing and grazing of farm animals.

Sample collection and identification. A total of 1485 ticks were collected from March to December, with peaks in May–June, during the years 2007–2008, from 80 dogs, 3 wild boars (*Sus scrofa meridionalis*), 36 sheep, 41 goats, 7 horses, 13 cattle, 1 deer (*Cervus elaphus corsicanus*) and 2 hedgehogs (*Erinaceus europaeus italicus*). Resident wild animals sampled during the study (deer, hedgehogs, wild boar) had been brought dead to our laboratories for necropsy analyses and tick capture. The other animals (dogs, sheep, goats, cattle and horses) from which ticks were removed came from farms, and the owners had provided the ticks using containment material. Ticks were removed from their host with tweezers and placed in vials with 70 % ethanol at room temperature. Identification was carried out by observation with a binocular microscope (× 10–50), and ticks were classified into family, genus and species using the taxonomic keys and morphometric tables available for tick identification (Manilla, 1998).

DNA extraction and PCR assay. All adult ticks belonging to the same species and collected from the same animal were segregated into pools of five ticks each. The pooled samples were immersed in distilled water for 10 min, dried on sterile filter paper, and crushed with a sterile scalpel in Eppendorf tubes. DNA extraction was performed using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions.

PCR was performed to evaluate the presence of *Rickettsia* species, *E. canis*, *A. phagocytophilum*, *C. burnetii*, *Bartonella* species and *Leishmania* species DNA in each pool using the GeneAmp PCR System 9700 (Applied Biosystems). The assay amplifies specifically a 500 bp fragment of the *ompB* gene (F: 5′-CTAGTGCAGATGCAAATG-3′; R: 5′-GTTTGAAATGATAATTG-3′) of *Rickettsia* SFG (Noda *et al.*, 1997), 200 bp of the *p30* gene (F: 5′-CATGATTGGGATGGAAGTCCAATAC-3′; R: 5′-ATGGCTGCCGATGTGTGATG-3′) of *E. canis* (Stich *et al.*, 2002), 293 bp of the 16S rRNA gene (F: 5′-TGTAGGCGGTTCGGTAAGTTAAAG-3′; R: 5′-CTTAACGCGTTAGCTACAACACAG-3′) of *A. phagocytophilum* (Kolbert, 1996), 257 bp of the superoxide dismutase gene (F: 5′-ACTCAACGCACTGGAACCGC-3′; R: 5′-TAGCTGAAGCCAATTCGCC-3′) of *C. burnetii* (Stein & Raoult, 1992), 298 bp of the 16S rRNA gene (F: 5′-GAGATGGCTTTTGGAGATTA-3′; R: 5′-CCTCCTTCAGTTAGGCTGGG-3′) of *Bartonella* species (Sander *et al.*, 1999) and 358 bp of the SSU rRNA gene (F: 5′-TCCCATCGCAACTTCGGT-3′; R: 5′-AAAGCGGGCGCGGTGCTG-3′) of *Leishmania* species (van Eys *et al.*, 1992).

Positive control DNAs were extracted from *C. burnetii* (Nine mile/I/EP1), *E. canis* (ATCC-CRL-10390), *B. henselae* (Houston 1 ATCC 49882), *Rickettsia conorii* (SIMKO EP7) and *L. infantum* (MON 1). Human promyelocytic leukaemia (HL60) cells infected with *A. phagocytophilum* were used as a positive control. Water samples were included in all amplifications as a negative control. PCR products were resolved on a 1–1.5 % agarose gel in 1 × TAE buffer (0.04 M Tris/acetate, 0.001 M EDTA). After electrophoresis at 100 V for 60 min, gels were stained with ethidium bromide and examined over UV light.

Data analysis. In order to verify the repeatability of our results, PCR analysis was performed three times on each tick pool. Infection rate in tick pools was estimated using the formula maximum-likelihood estimation (MLE)$=1-(1-Y/X)^{1/m}$ as described by Walter *et al.* (1980), where Y=number of positive pools, X=number of pools and m=number of organisms per pool. This formula assumes that when a PCR product is positive from a pool of five ticks, only one tick in the pool is considered to be infected.

RESULTS

Tick species and host distribution

Seven species of ticks belonging to the order Ixodidae were identified among a total of 1485 adult ticks randomly collected from mammals in Sardinia: *Rhipicephalus sanguineus*, *Rhipicephalus turanicus*, *Rhipicephalus bursa*, *Rhipicephalus pusillus*, *Hyalomma marginatum marginatum*, *Haemaphysalis sulcata* and *D. marginatus*. The number of ticks collected from animals and used in this study were as follows: 970 from dogs; 180 from sheep; 205 from goats; 65 from cattle; 35 from horses; 10 from hedgehogs; 5 from deer; and 15 from wild boars. The percentages of tick species abundance in Sardinia are reported in Table 1.

A total of 92.3 % of *Rhipicephalus sanguineus* tick species were removed from dogs. *Rhipicephalus turanicus* ticks were removed mostly from sheep and goats (32.7 % and 51.7 %, respectively). *Rhipicephalus bursa* specimens occurred mostly on sheep (38.9 %), but were also collected from a wide range of other hosts: 33.3 % from horses; 16.7 % from goats; 5.5 % from cattle; and 5.5 % from deer. *Rhipicephalus pusillus* ticks were found only in a hedgehog. Fifteen ticks identified as *Hyalomma marginatum marginatum* were removed from cattle. *Haemaphysalis sulcata* ticks were all detected in goats and sheep. Finally, ticks removed from wild boars were classified as *D. marginatus* (1 %). Tick association with mammal hosts is reported in Table 2.

Detection of pathogens in ticks

Six out of the seven tick species identified contained DNA of pathogens. Out of 209 pools of *Rhipicephalus sanguineus*, nine pools (eight from dogs and one from sheep) were positive for *Rickettsia* SFG; one pool from a dog was positive for *E. canis*, five pools (four from dogs and one from a goat) were positive for *C. burnetii*, and one pool from a dog was positive for *Bartonella* species. Twelve

Table 1. Number of identified total tick pools, percentage of their estimated abundance with respect to the total number of ticks collected, pools testing positive for *Rickettsia* species, *E. canis*, *A. phagocytophilum*, *C. burnetii* and *Bartonella* species, and the rate of infection (RI) in pools from each tick species

Tick species	No. of pools	Tick abundance (%)	Positive pools				
			Rickettsia (SFG)	*E. canis*	*A. phagocytophilum*	*C. burnetii*	*Bartonella* spp.
Rhipicephalus sanguineus	209	70.4	9	1		5	1
RI (%)*			0.9	0.09		0.5	0.09
Rhipicephalus turanicus	58	19.5	12		5	1	2
RI (%)*			4.5		1.8	0.3	0.7
Rhipicephalus bursa	18	6.1					1
RI (%)*							1.1
Rhipicephalus pusillus	1	0.3	1				
RI (%)*			100				
Haemaphysalis sulcata	5	1.7		3		1	
RI (%)*				16.7		4.4	
Dermacentor marginatus	3	1		1			
RI (%)*				7.8			

*MLE$=1-(1-Y/X)^{1/m}$.

pools (five from sheep, five from goats, one from cattle and one from a horse) of *Rhipicephalus turanicus* were positive for *Rickettsia* SFG, five pools (one from sheep and four from goats) were positive for *A. phagocytophilum*, one pool from a goat was positive for *C. burnetii* and two pools (one from a dog and one from a goat) were positive for *Bartonella* species. One pool of *Rhipicephalus bursa* from a goat was positive for *Bartonella* species. The pool of *Rhipicephalus pusillus* from a hedgehog was positive for *Rickettsia* SFG. *Hyalomma marginatum marginatum* ticks were grouped in three pools, which tested negative for all pathogens considered in this study. *Haemaphysalis sulcata* ticks were grouped in five pools: three pools from goats were positive for *E. canis*, and one was positive for *C. burnetii*. Finally, positivity to *E. canis* was detected in one out of three *D. marginatus* pools, from a wild boar. No positivity to *Leishmania* species was detected. Data regarding the prevalence of tick infection by pathogens are summarized in Table 1.

DISCUSSION

In this paper, we report the results of a 2-year survey carried out in two areas of south-eastern Sardinia with the aim of evaluating tick distribution and circulation of micro-organisms in ticks collected from different mammals. *Rhipicephalus sanguineus* was confirmed to be the most represented tick species in Sardinia, accounting for over 70 % of all ticks examined. *Rhipicephalus sanguineus*, which is considered to be a dog-associated species and was found mostly on these animals also in this study, is well adapted to live in the mesomediterranean bioclimatic zone; as a consequence, it can feed in all stages, and generally does not require other host species to complete its life cycle (Psaroulaki *et al.*, 2006). *Rhipicephalus turanicus* is considered the species mostly associated with sheep (Genchi & Manfredi, 1999). This was in agreement with our study; in fact, most of the *Rhipicephalus turanicus* ticks were removed from small ruminants. *Rhipicephalus bursa* ticks were also found in this study. In the Mediterranean basin, this species is considered a major ectoparasite of sheep (Yeruham *et al.*, 2000), from which it was also recovered in high percentages in this work. However, although it was retrieved mostly from small ruminants, we found *Rhipicephalus bursa* in many other host species, which might act as vectors facilitating the spread of this tick species among flocks.

The *Rhipicephalus pusillus* ticks identified were all collected from a hedgehog; in fact, although these ticks are reported to inhabit rabbits, on which they feed during all stages, they can occasionally infest hedgehogs and rodents (Walker *et al.*, 2000), consistent with our findings. We also recovered *Hyalomma marginatum marginatum*, only from cattle and not from other host species. *Haemaphysalis sulcata* was detected only on small ruminants, in accordance with the studies carried out by Genchi & Manfredi (1999), who reported the frequent occurrence of these species on small ruminants when they are reared on pastures, mainly in central-southern Italy. All ticks identified as *D. marginatus* were found only in wild boars. These ticks require warm, dry habitats and inhabit wild boars, which constitute the main hosts (Ortuño *et al.*, 2006). *Ixodes marginatus* was not detected in this study. However, this is not surprising, since it has been previously reported as a tick species scarcely present in Sardinia (Alberti *et al.*, 2005a).

Table 2. Association between different tick species and the mammals from which they were collected

Tick species	Total ticks	Total pools	Mammalian host
Rhipicephalus sanguineus	965	193	Dog
	45	9	Sheep
	20	4	Goat
	10	2	Cattle
	5	1	Hedgehog
Total	**1045**	**209**	
Rhipicephalus turanicus	5	1	Dog
	95	19	Sheep
	150	30	Goat
	35	7	Cattle
	5	1	Horse
Total	**290**	**58**	
Rhipicephalus bursa	35	7	Sheep
	15	3	Goat
	5	1	Cattle
	30	6	Horse
	5	1	Deer
Total	**90**	**18**	
Rhipicephalus pusillus	5	1	Hedgehog
Total	**5**	**1**	
Haemaphysalis sulcata	5	1	Sheep
	20	4	Goat
Total	**25**	**5**	
Hyalomma marginatum marginatum	15	3	Cattle
Total	**15**	**3**	
Dermacentor marginatus	15	3	Wild boar
Total	**15**	**3**	
Overall total	**1485**	**297**	

This study provides data regarding the prevalence of *Rickettsia* species, *E. canis*, *A. phagocytophilum*, *C. burnetii*, *Bartonella* species and *Leishmania* species potentially transmitted by ticks in Sardinia. According to species-specificity in tick behaviour, Ixodida ticks of the genera *Rhipicephalus*, *Dermacentor*, *Ixodes* and *Amblyomma* are the most important vectors of *Rickettsiae* species including human rickettsial pathogens (Duh *et al.*, 2006).

Ticks of the family Ixodidae can transmit *Anaplasma* species and *Ehrlichia* species, which are closely related to the genus *Rickettsia*. *Rhipicephalus sanguineus* is also the primary vector of *E. canis* (Murphy *et al.*, 1998); this observation was confirmed in this study. Here, PCR evidence of *E. canis* was detected not only in *Rhipicephalus sanguineus* but also in *Rhipicephalus turanicus*, *Haemaphysalis sulcata* and *D. marginatus*. Other authors have found PCR evidence of *E. canis* in pools of *Rhipicephalus sanguineus* collected from Venezuela (Unver *et al.*, 2001), Albania (Christova *et al.*, 2003) and Oklahoma (Murphy *et al.*, 1998). The detection of five *A. phagocytophilum*-positive pools in *Rhipicephalus turanicus* collected from small ruminants was in accordance with the studies carried out by Keysary *et al.* (2007). However, we did not find PCR-positivity for *A. phagocytophilum* in *Rhipicephalus sanguineus* (Alberti *et al.*, 2005a), *D. marginatus* or *Rhipicephalus bursa* (Merino *et al.*, 2005), although this might be due to the sample size or to the different geographical areas where ticks were collected. However, our finding of *A. phagocytophilum* positivity also in other tick species parasitizing different animal hosts to those previously reported is a relevant finding for human and animal health (Alberti *et al.*, 2005b; Ruscio & Cinco, 2003; Mastrandrea *et al.*, 2006).

In this work, *Rhipicephalus sanguineus*, *Rhipicephalus turanicus* and *Haemaphysalis sulcata* were positive for *C. burnetii*. This pathogen is reported to be carried by several tick species: *Dermacentor* species in Germany (Sting *et al.*, 2004), plus *Rhipicephalus sanguineus* and *Hyalomma*

species ticks in Cyprus (Spyridaki *et al.*, 2002). Infection by *C. burnetii* in *Rhipicephalus turanicus* was detected in the Greek island of Cephalonia (Psaroulaki *et al.*, 2006).

It is known that *Rhipicephalus sanguineus* ticks could be potential vectors of *Bartonella* species, as has been hypothesized since 1992 (Lucey *et al.*, 1992). Experimental vector transmission studies must be performed to validate the hypothesis that ticks transmit *Bartonella* species to animals and humans (Billeter *et al.*, 2008). In our work, not only *Rhipicephalus sanguineus*, but also *Rhipicephalus turanicus* and *Rhipicephalus bursa*, showed positivity for *Bartonella* species.

We did not find positivity to *Leishmania* species in all seven tick species analysed. The vectorial competence of *Rhipicephalus sanguineus* in relation to the biology of *Leishmania* and to the epidemiology of canine leishmaniasis is strongly questionable, taking into account the strict association of this tick species with dogs and the low indices of natural leishmanial infection as presented by Coutinho *et al.* (2005).

These results increase our knowledge of tick-borne diseases in Sardinia, and provide a useful contribution to understanding their epidemiology. These findings might be helpful for evaluating patient health problems and for risk prevention, and could provide the basis for a plan aimed at monitoring the spread of tick-borne diseases in the island.

ACKNOWLEDGEMENTS

The authors thank Dr Sandro Rolesu for data analysis, and Dr Maria Filippa Addis for her critical reading and suggestions on the manuscript.

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