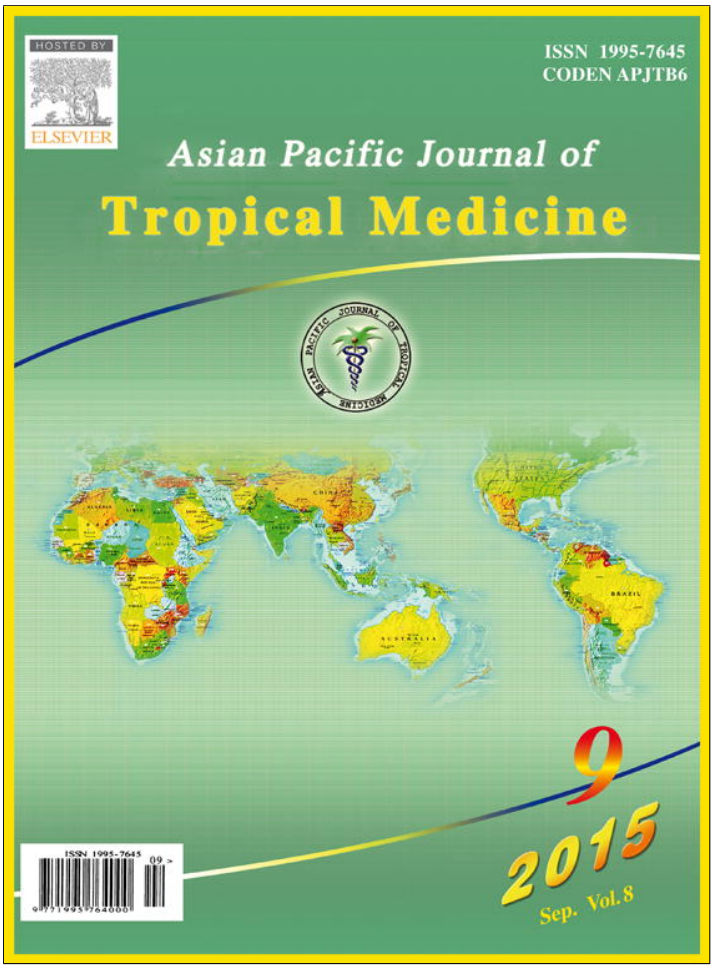

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Molecular survey of tick-borne pathogens in Ixodid ticks collected from hunted wild animals in Tuscany, Italy

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ABSTRACT

Objective: To determine the prevalence of zoonotic tick-borne bacteria in feeding ticks removed from hunted wild animals.
Methods: PCR was executed on DNA extracted from 77 tick pools to detect *Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia burgdorferi* sensu lato, *Coxiella burnetii* and *Rickettsia* spp.
Results: A total of 432 ticks were collected: 30 (6.94%) *Haemaphysalis punctata*, 72 (16.7%) *Dermacentor marginatus* and 330 (76.38%) *Ixodes ricinus*. For each animal one or two pools of 3 ticks of the same species was constituted. Seventy-seven tick pools were examined by PCR: 58 (75.32%) resulted infected and among them 14 (18.18%) showed co-infections. In particular, 29 (37.66%) pools were positive for *Bartonella* spp., 23 (29.87%) for *Anaplasma phagocytophilum*, 16 (20.78%) for *Rickettsia* spp., and 5 (6.49%) for *Borrelia burgdorferi* s.l. All samples were negative for *Coxiella burnetii*.
Conclusions: The results demonstrate the presence of several zoonotic tick-borne pathogens in the studied area, and underline the risk of exposure to infections for hunters not only during the outdoor activity, but also when they manipulate hunted animals infested by infected ticks.

1. Introduction

Tuscany is a region in central Italy characterized by the presence of wide mountain, hill and plain areas, rich in vegetation, that are frequently used for recreational activity, mainly hunting, mushrooms and chestnuts collecting, and walking.

These areas are home to several species of wild animals that are frequently infested by ticks belonging to different species. The sheep tick *Ixodes ricinus* (*I. ricinus*) is the most common hard tick species found in these areas, as well as in the rest of Italy, but *Dermacentor* sp. [*Dermacentor marginatus* (*D. marginatus*)], *Hyalomma* sp., *Haemaphysalis* sp., *Rhipicephalus* sp. are present too.

These hematophagous arthropods are often vectors of several tick-borne agents. Among them, some bacteria are able to infect wild and domestic animals, and humans in which may determine severe clinical forms. Lyme disease by *Borrelia burgdorferi* (*B. burgdorferi*) sensu lato spirochetes, granulocytic anaplasmosis by *Anaplasma phagocytophilum* (*A. phagocytophilum*), and rickettsiosis due to rickettsiae belonging to the Spotted Fever Group are well known in human medicine [1].

Bartonellosis is a zoonotic vector-borne disease, that is traditionally associated to *Bartonella henselae*, etiologic agent of the Cat Scratch Disease. However, the genus *Bartonella* includes several species able to infect humans [2].

Coxiella burnetii (*C. burnetii*), responsible for Q Fever, is transmitted through ingestion of contaminated food, mainly dairy products, or inhalation of infected aerosol, but infection due to infected ticks is possible, mainly in areas where the arthropod population is abundant [3].

In Italy, investigations on the prevalence of tick-transmitted pathogens have been previously conducted testing ticks collected in wild and urban/periurban habitats [4]. Hunters are at high risk of exposure to tick bites, because they frequent habitat infested by arthropods, but they can be attached by ticks also during manipulation of the carcasses of hunted animals.

The aim of the present study was to determine the prevalence of the main zoonotic bacterial agents transmitted by hematophagous arthropods, in particular *A. phagocytophilum*, *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii* and *Rickettsia* spp.,

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in ticks removed from hunted wild animals living in mountain and hilly areas of Tuscany, central Italy, frequented by visitors for hunting and other recreational activities.

2. Materials and methods

2.1. Study sites and sampling

Feeding ticks were collected from 72 wild animals, in particular 30 fallow deer (*Dama dama*), 29 red deer (*Cervus elaphus*), 7 wild boars (*Sus scrofa*), and 6 roe deer (*Capreolus capreolus*).

The animals lived in mountain and hilly areas of Tuscany, rich of mixed forests mainly composed by beech (*Fagus sylvatica*), chestnut (*Castanea sativa*), fir (*Abies alba*), oak (*Quercus ilex* and *Quercus robur*). Several different bird species live usually in these areas, as well as various large and small mammals such as roe deer, fallow deer, red deer, wild boar, fox (*Vulpes vulpes*), wolf (*Canis lupus*), hare (*Lepus lepus*), marmot (*Marmota marmota*), European badgers (*Meles meles*), hedgehog (*Erinaceus europaeus*), also different rodent species can be found there. Some zones of these areas host farm animals, in particular horses employed for trekking, cattle and sheep. Dogs with their owners are frequently present for hunting activity.

Only adult (male and female) ticks were collected. Six ticks were removed from each animals and placed into sterile tubes. Samples were transferred to the laboratory and kept frozen at −20 °C. All ectoparasites were stored on ice during the identification procedure, which was done on the basis of their morphologic features by using standard taxonomic keys [5].

2.2. Molecular analysis

2.2.1. DNA extraction

DNA was extracted from each pool constituted by 3 adult ticks belonging to the same species and collected from the same animal. All tick pools were disinfected by immersion into a 70% ethanol solution for 5 min, and then rinsed with sterile phosphate buffered saline. Successively each tick was cut with sterile scalpel and 100 μL of sterile phosphate buffered saline and 20 μL of proteinase K were added.

DNA was extracted using the DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and stored at 4 °C until used as template for PCR assays.

2.2.2. Polymerase chain reaction

PCR assays to detect DNA of *A. phagocytophilum*, *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii*, *Rickettsia* spp. were performed in thermal cycler (Gene-Amp PCR System 2700, Perkin–Elmer, Norwalk, Connecticut, USA) using the EconoTaq PLUS 2 × Master Mix (Lucigen Corporation, Middleton, Wiskonsin, USA).

Sterile distilled water instead of DNA was included as negative control to ensure the absence of contamination in each reaction mixture. Genomic DNA, obtained from immunofluorescent slides (Fuller Laboratories Fullerton, California, USA) for each pathogen, was used as positive control.

A. phagocytophilum: A nested PCR was carried out using the primers GE3a and GE10r for the primary reaction which amplifies a 932 bp fragment of the 16S rRNA gene of *A. phagocytophilum*, and the primers GE9f and GE2 for the secondary assay, which amplified a 546 bp fragment of the same gene [6].

Bartonella spp.: DNA samples were employed in a PCR assay to identify the *Bartonella* genus. The primers p24E and p12B, previously described by Relman *et al.* (1990) [7], were used in this protocol to amplify a 296 bp fragment of the *Bartonella* 16S rRNA gene.

B. burgdorferi s.l.: Primers JS1 and JS2 were used to amplify a 261 bp fragment of the 23S rRNA gene of *B. burgdorferi* s.l. [8].

C. burnetii: *C. burnetii* was identified by amplifying a 687 bp fragment of the *IS1111a* gene using primers Trans-1 and Trans-2 as described by Berri *et al.* (2009) [3].

Rickettsia spp.: PCR with the primers Rr190.70p and Rr190.701 were carried out to amplify a 632 bp fragment of the gene encoding the outer surface protein ompA of *Rickettsia* spp. as described by Roux *et al.* (1996) [9]. Since this protocol does not allow detecting *Rickettsia helvetica*, *Rickettsia akari*, *Rickettsia australis*, and *Rickettsia bellii*, a second PCR assay was performed using the primers RpCS.877p and RpCS.1258n which amplify a 381 bp fragment of the *gltA* gene [10].

All the amplification products were analyzed by electrophoresis on 1.5% agarose gel at 100 V for 45 min; gel was stained with ethidium bromide and observed. GelPilot 100 bp Plus Ladder (Qiagen) was used as DNA marker.

2.2.3. Sequencing

Bartonella spp. and *Rickettsia* spp. amplicons were sent to PRIMM (Milano, Italy) for DNA sequencing; the nucleotide sequences were compared with those present in GenBank database using the basic local alignment search tool (BLAST).

3. Results

A total of 432 ticks were collected and resulted belonging to the species *Haemaphysalis punctata* (*H. punctata*) (*n* ticks = 30, 6.94%), *D. marginatus* (*n* = 72, 16.7%) and *I. ricinus* (*n* = 330, 76.38%). For each animal one pool was constituted with 3 ticks of the same species. For five animals two pools were obtained. A total of 77 tick pools (10 for *H. punctata*, 12 for *D. marginatus*, 55 for *I. ricinus*) was examined by PCR. Fifty-eight (75.32%) pools resulted infected, and among them 14 (18.18%) showed co-infections (Table 1).

A. phagocytophilum was detected in 23 (29.87%) tick pools. More in detail, the DNA was found in 22 *I. ricinus* pools

Table 1
Co-infections detected in the examined tick pools.

Pathogens	Positive samples (*n*)	Tick species
A. phagocytophilum + *B. burgdorferi*	2	*I. ricinus*
A. phagocytophilum + *Bartonella* spp.	4	3 *I. ricinus*, 1 *H. punctata*
Bartonella spp. + *B. burgdorferi*	1	*D. marginatus*
R. monacensis + *B. burgdorferi*	1	*I. ricinus*
R. monacensis + *Bartonella* spp.	4	*I. ricinus*
A. phagocytophilum + *B. burgdorferi* + *Bartonella* spp.	1	*I. ricinus*
A. phagocytophilum + *Bartonella* spp. + *R. monacensis*	1	*I. ricinus*

Table 2
Bartonella species detected in tick pools in relation to the animal source.

Bacteria	Tick species (*n*)	Animal species
Bartonella acomydis	*I. ricinus* (2)	Red deer
Bartonella bacilliformis	*I. ricinus* (2)	Red deer
	I. ricinus (1)	Fallow deer
	H. punctata (1)	Fallow deer
B. bovis	*I. ricinus* (3)	Red deer
	I. ricinus (3)	Fallow deer
	D. marginatus (1)	Red deer
	H. punctata (1)	Red deer
	H. punctata (2)	Fallow deer
B. chomelii	*I. ricinus* (5)	Red deer
	I. ricinus (3)	Roe deer
	D. marginatus (1)	Roe deer
Bartonella tribocorum	*I. ricinus* (2)	Red deer
Bartonella vinsonii berkoffii	*I. ricinus* (2)	Red deer

Table 3
Rickettsia species detected in tick pools in relation to the animal source.

Bacteria	Tick species (*n*)	Animal species
R. monacensis	*I. ricinus* (8)	Fallow deer
	H. punctata (2)	Fallow deer
	I. ricinus (4)	Red deer
R. slovaca	*D. marginatus* (2)	Red deer

collected from 11 fallow deer, 8 red deer, 2 roe deer, and 1 *H. punctata* pool collected from a fallow deer.

Twenty-nine (37.66%) tick pools were positive to amplification of 16S rRNA gene of *Bartonella* genus. Sequencing analysis identified 2 (2.6%) *Bartonella acomydis*, 4 (5.2%) *Bartonella bacilliformis*, 10 (12.98%) *Bartonella bovis* (*B. bovis*), 9 (11.68%) *Bartonella chomelii* (*B. chomelii*), 2 (2.6%) *Bartonella tribocorum*, 2 (2.6%) *Bartonella vinsonii* subsp. *Berkoffii* (*B. vinsonii* subsp. *berkoffii*). Table 2 shows the tick species in which *Bartonella* DNA was found and the animals from which ticks were collected.

B. burgdorferi s.l. DNA was found in 5 (6.49%) tick pools: 4 *I. ricinus* pools from fallow deer, and 1 *D. marginatus* pool from a red deer. All tick pools resulted negative for *C. burnetii*.

Rickettsia infection was detected in 16 (20.78%) samples; in particular *Rickettsia monacensis* (*R. monacensis*) was found in 14 (18.18%) pools, and *Rickettsia slovaca* (*R. slovaca*) in 2 (2.6%). Positive tick species and animal source are reported in Table 3.

4. Discussion

The present study has been conducted on ticks collected from wild animals captured by hunters in areas largely frequented for hunting and other recreational activities. Among the tick species found, *I. ricinus* was the most frequently detected (76.38%), confirming its wide distribution in wild environment. A total of 77 tick pools were examined by PCR and a high percentage (75.32%) of positive results was obtained. Dual and triple infections were recognized in 18.18% of cases, suggesting the potential risk of multiple infections from a single tick bite.

A relevant prevalence (37.66%) for Bartonellosis was observed. A recent study conducted in questing ticks collected in an urban park in Rome, Italy, has not found *Bartonella* spp. [4], but other investigations carried out in Italy and in the rest of Europe have detected *Bartonella* spp. DNA in hematophagous arthropods [11,12].

B. bovis and *B. chomelii* were present in the highest number of the examined tick pools. Cattle constitute the reservoir of these species that usually do not cause clinical signs. However, *B. bovis* was identified as a cause of bovine endocarditis and long lasting bacteremia. In Europe, *B. bovis* infections in cattle have been reported in France [13,14], Italy [15] and Poland [16], whereas *B. chomelii* is considered the most frequent species infecting cattle in Spain [17].

The pathogenic role of the other detected *Bartonella* species has not been fully determined, however all bartonellae are considered potential pathogens for animals and humans [2].

A. phagocytophilum was found in 29.87% of samples. This is a predictable result, because this pathogen has been frequently detected in central Italy that is considered an endemic area. Moreover, the obtained results confirm that *I. ricinus* is one of the most important vectors of this pathogen in Europe, and wild ruminants represent its reservoirs [18].

The 20.78% of examined tick pools were positive for *Rickettsia* spp. In particular, two species belonging to the Spotted Fever Group were found: *R. monacensis* and *R. slovaca*.

R. monacensis is distributed all over Europe and vector mainly by *I. ricinus*, as demonstrated by our survey too. The prevalence of tick infection reaches 34.6% in some zones of Europe. Human cases with a clinical picture similar to that caused by the Mediterranean Spotted Fever by *Rickettsia conorii*, have been associated to *R. monacensis* [19].

R. slovaca, which causes a human clinical syndrome named tick-borne lymphadenopathy or *Dermacentor*-borne necrosis-erythema-lymphadenopathy characterized by inoculation eschar on the scalp and enlarged cervical lymph nodes [20], has been detected in *D. marginatus* and *H. punctata* ticks collected from herbivores in Sicily, south Italy [21], and in ticks *D. marginatus* removed from humans in Tuscany [22]. In this study, *R. slovaca* DNA was found in 2 pools of *D. marginatus* collected from red deer, confirming this tick as the main vector.

C. burnetii has not been detected during the present investigation. This result could mean that the Q fever agent was not present in the geographic areas in which ticks had been sampled, or that, despite the presence, it was not spread in arthropod population. However, other recent studies carried out in ticks and animals from central Italy have found *C. burnetii* DNA, suggesting that the pathogen is circulating in this geographic area [4,23].

In Italy, the reported *B. burgdorferi* s.l. prevalence in questing ticks varies considerably among the study areas: from 1.3% to 40.0% in northern Italy, from 8.7% to 30.0% in central Italy [4]. Different results are related to differences in environmental features, climatic conditions and collected tick samples (number, species, and stage). In our study, in which only adult ticks directly collected from hunted animals were tested, 6.49% prevalence was found. This value is not high, but still confirms the circulation of *B. burgdorferi* s.l. in the studied geographic area.

In conclusion, findings of the present survey underline the potential risk of transmission of zoonotic tick-borne pathogens to humans, which for recreational or work activities frequent rural and forestry environments. Hunters are generally exposed to risk of tick bites during their outdoor activity, but in particular when they manipulate the carcasses of hunted animals. In fact,

ticks quite rapidly leave the killed animals going to the surrounding environment and on present people.

The circulation of arthropod-borne pathogens is not only of public health concern, but also of veterinary interest. In fact, several tick-borne bacteria are able to infect domestic animals (horse, cattle, dogs), which share the same environment with wild animals, causing diseases varying from asymptomatic to severe forms.

Conflict of interest statement

We declare that we have no conflict of interest.

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