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# Detection of potentially pathogenic bacteria from *Ixodes ricinus* carried by pets in Tuscany, Italy

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## ABSTRACT

**Background** Ticks are vectors of disease-causing pathogens that pose a serious threat to animals and people. Dogs and cats are exposed to tick infestation in multiple ways and can easily transport infected ticks into domestic environments and potentially transfer them to people. Pet owners are at increased risk of picking up ticks from their pets and developing tickborne diseases. This study aims to detect the presence of pathogens of potential public health interest in ticks removed from cats and dogs in Tuscany, Italy.

**Methods** The collected ticks were screened for the presence of protozoan (*Theileria* species and *Babesia* species) and bacterial (*Rickettsia* species, *Anaplasma* species, *Ehrlichia* species, *Chlamydia* species, *Bartonella* species and *Coxiella burnetii*) pathogens using PCR.

**Results** PCR and sequencing analysis revealed that 3 per cent of the ticks were PCR-positive for the presence of *Rickettsia helvetica* DNA, 5 per cent of ticks were PCR-positive for *Bartonella henselae* DNA, and 46 per cent of ticks were PCR-positive for *Chlamydia psittaci* and *Chlamydia abortus* DNA. None of the examined ticks was PCR-positive for *Theileria* species, *Babesia* species, *Anaplasma* species, *Ehrlichia canis* or *Coxiella burnetii* DNA.

**Conclusion** The results of this preliminary study highlight the importance of monitoring companion animals as indicators to evaluate the health status of their owners. Preventive measures are necessary to limit the spread of zoonotic pathogens from companion animals to people within the home environment.

## INTRODUCTION

Ticks vector a wide variety of pathogens, including bacteria, viruses, protozoa and fungi, most of which have medical and veterinary importance.[1] The range of emerging tickborne diseases (TBDs) affecting animals and people has been increasing in recent years, and physicians and veterinarians are focusing their attention on the management of these diseases.[2]

Climate change and landscape modification as a result of human activity influence the abundance and distribution of tick species, and consequently the emergence/re-emergence of TBDs.[3 4] Companion animals that spend some time outdoors in peridomestic settings could be accidental hosts for several tick species and serve as a reservoir for some tickborne pathogens that can also affect people.[3] In this context, pets can play a role in the spread of tickborne pathogens in urban areas and contribute to the epidemiology of TBDs in people.

Italian tick fauna comprises more than 40 species, making it one of the most diverse across Europe.[5] *Ixodes ricinus*, a three-host tick, is an important vector of Lyme disease as well as of other diseases of medical and veterinary importance.[6] This tick species is the most abundant and widespread in central and northern Italy, in urban and periurban areas, and in all areas where humidity and temperature offer favourable environmental conditions for its development.[7–10]

The objective of this study was to evaluate both the presence of *I ricinus* ticks on cats and dogs presented at a veterinary clinic in central Italy and the pathogens associated with these ticks.

## MATERIALS AND METHODS

### Study area

Ticks were collected from infested dogs and cats from Le Grazie, a locality 20 km from Pistoia in central Italy. This area includes different habitat types, such as mountains, flat valleys and hills that are rich in farmlands, fields and grassland that are frequently used for human recreational activity.

### Study design and tick collection

In September 2018, ticks were collected from 17 pets (two dogs and 15 cats) seen at a private veterinary clinic after parasite infestation. As reported by their owners, pets spent time outdoors and were kept indoors during the night. The pets showed no apparent clinical signs at the time of sampling.

All ticks found on the pets' coats were removed with tweezers and placed in vials with 70 per cent ethanol at room temperature for submission to the laboratories of Experimental Zooprofilattico Institute of Sardinia. The ticks were then examined under a microscope and classified into family, genus and species using available taxonomic keys and morphometric tables.[11]

### DNA extraction

After microscopic identification, ticks were immersed in distilled water for 10 minutes, dried with sterile filter paper and crushed with a sterile scalpel in Eppendorf tubes (Eppendorf, Hilden, Germany). DNA was then extracted from each of the ticks using the DNeasy Blood & Tissue Kit (QIAGEN, Chatsworth, California, USA) according to the manufacturer's instructions. DNA was stored at 4°C until use in PCR amplification assays.

### Detection and characterisation of tickborne pathogens

The DNA samples from individual ticks were screened for the presence of *Rickettsia* species, *Anaplasma* species, *Ehrlichia canis*, *Coxiella burnetii*, *Bartonella* species, *Chlamydia* species and *Babesia/Theileria* species DNA using PCR. DNA extracted from uninfected ticks was used as a negative control and DNAs from *Rickettsia massiliae*, *Anaplasma phagocytophilum*, *E canis*, *Babesia bovis*, *Chlamydia suis*, *Bartonella henselae* and *Coxiella burnetii* were used as a positive control. The PCR primers and cycling conditions used were the same as those documented in previous studies.[12–14]

The reactions were performed in automated DNA thermal cyclers (GeneAmp PCR System 9700; Applied Biosystems, Courtaboeuf, France). PCR products were analysed by electrophoresis on 1.5 per cent agarose gels stained with SYBR Safe DNA Gel Stain (Thermo Fisher) and examined under UV transillumination. The PCR products were then purified using the QIAquick Spin PCR Purification Kit (QIAGEN).

### Sequencing assays

All purified PCR products were sequenced in both directions using a DNA sequencing kit (dRhodamine Terminator Cycle Sequencing Ready Reaction; Applied Biosystems) and an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California, USA), used according to the manufacturer's instructions.

### BLASTn and phylogenetic analyses

Chromatograms of forward and reverse sequences were edited with Chromas 2.2 (Technelysium, Helensvale, Australia) and then aligned with ClustalX[15] to assign them to unique sequence types. The sequences were then checked against the GenBank database using nucleotide Basic Local Alignment Search Tool (BLASTn).[16] Pairwise/multiple sequence alignments and sequence similarities were calculated using ClustalW[17] and the identity matrix options of BioEdit,[18] respectively. Evolutionary analyses were conducted in MEGA6[19] using the maximum likelihood method based on models identified as the best with the same software.[20 21] The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed.[22]

### Sequence accession numbers

The sequences of *Rickettsia*, *Bartonella* and *Chlamydia* species obtained in this investigation were deposited in the GenBank database with accession numbers MN209226–MN209227 (*B henselae* 16S rRNA), MN203727–MN203728 (*C psittaci* and *C abortus* 16S rRNA) and MN226407 (*Rickettsia glt*A). All sequences were submitted using the National Center for Biotechnology Information (Bethesda, Maryland) BankIt V.3.0 submission tool (http://www3.ncbi.nlm.nih.gov/BankIt/).

## RESULTS

### Tick morphological identification and tickborne pathogens

A total of 37 ticks were identified by morphological keys as *I ricinus*, as shown in table 1. BLAST analyses of the PCR products obtained from these ticks revealed that one (3 per cent) rickettsial gltA gene sequence was identical to the top BLAST hit *Rickettsia helvetica* (KY488349) previously obtained from wild rodents in Poland.

Seventeen ticks (46 per cent), collected from 12 cats, were positive for the presence of chlamydial DNA. In particular, 16 sequences shared a unique sequence type that shared 100 per cent nucleotide identity with *C abortus* 16S rRNA previously reported from a dolphin (MH842741).

BLAST comparisons revealed that DNA from one tick from one cat had 100 per cent similarity with *C psittaci* strains collected from sheep, birds and cattle (CP041038, CP033059 and CP047319, respectively), indicating that the sequences detected potentially belong to strains related to this previously described chlamydial species. One sequence obtained from one tick showed 100 per cent identity with a *C psittaci* strain collected from birds (HQ616171) and identified as the closest match by nBLAST.

Two *I ricinus* ticks collected from one cat and one dog were positive for the presence of *Bartonella* species 16S rRNA gene DNA that shared 100 per cent similarity with *B henselae* strains isolated from people and cats (KY773227, KF419277, JN646650).

None of the collected ticks was positive for *Anaplasma* species, *E canis*, *Coxiella burnetii* or *Babesia/ Theileria* species.

Mixed infections with *B henselae/C abortus and B henselae/C psittaci* were recorded in two ticks.

### Phylogenetic analyses

The phylogenetic study of the partial *glt*A gene was performed by aligning the sequence type Seq1Ric (found in one *I ricinus* tick from one cat) with selected *Rickettsia* reference sequences. Seq1Ric was located in the same clade as an *R helvetica* strain belonging to the spotted fever group *Rickettsiae* (figure 1).

**Table 1** Number of tick species analysed, hosts, positive ticks tested for pathogens by PCR assays and BLAST analysis

| Tick species (n) | Animal hosts (n) | Sex and engorgement status (n) | Pathogen detected (number of positive ticks) | BLAST analysis |
|---|---|---|---|---|
| *Ixodes ricinus* (37) | Cats (15) | Female PE (18) | *Bartonella henselae* (1) | *B henselae* (100%) isolated from people and cats from Korea, France and New Caledonia. |
| | | | *Rickettsia helvetica* (1) | *R helvetica* (100%) isolated from wild rodents and ticks from Poland, Russia and Hungary. |
| | | | *Chlamydia abortus* (9) | *C abortus* (100%) isolated from dolphins and sheep from Italy and the USA. |
| | | Female E (8) | *Chlamydia abortus* (2) | |
| | | Female NE (4) | *Chlamydia abortus* (4) | |
| | | Male NE (4) | *Chlamydia abortus* (1)<br>*Chlamydia psittaci* (1) | *C psittaci* (100%) from birds, sheep and cattle from Russia and the Faroe Islands. |
| | Dogs (2) | Female NE (2) | – | – |
| | | Female PE (1) | *Bartonella henselae* (1) | *B henselae* (100%) isolated from people and cats from Korea, France and New Caledonia. |

BLAST, Basic Local Alignment Search Tool; E, engorged; NE, not engorged; PE, partially engorged.

On sequencing and ClustalX alignment, 16S rRNA sequences were assigned to two different sequence types: Seq1Chl (derived from 16 sequences from *I ricinus* ticks from 12 cats) and Seq2Chl (derived from one *I ricinus* tick from one cat). The phylogenetic tree showed that the Seq1Chl and Seq2Chl sequence types were included in two strongly supported monophyletic clades with *C abortus* and *C psittaci* reference sequences, respectively (figure 2).

The unique sequence type Seq1Bart, obtained from the two *Bartonella*-positive sequences, was found to group with a reference strain representative of *B henselae* (figure 3).

## DISCUSSION

This survey aimed to investigate the presence of ticks on dogs and cats living in an urban area near Pistoia, Tuscany (central Italy) and evaluate the pathogens of public health significance associated with these ticks. *I ricinus* was the only tick species detected on these pets, which is consistent with other studies carried out in central, northern and southern Italy that found that *I ricinus* is widely distributed through the Italian regions and dogs and cats are common hosts.[23–27] Previous studies confirmed that *I ricinus* was the most abundant and common tick species

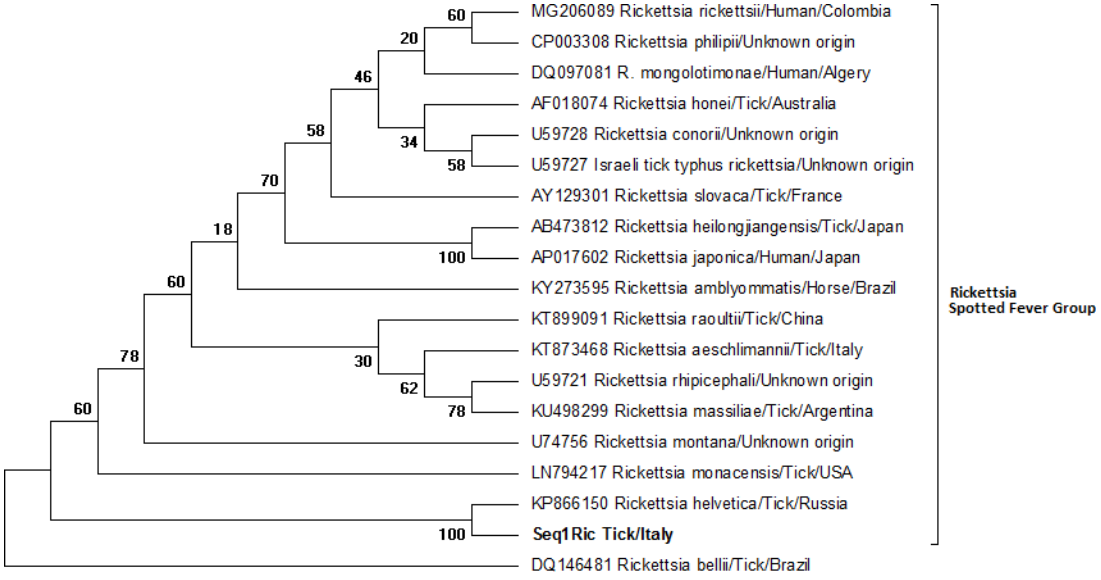


**Figure 1** Phylogenetic tree of *Rickettsia helvetica* detected in ticks based on partial *gltA* sequence gene. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura three-parameter model. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 711 positions in the final data set. The numbers at the nodes indicate bootstrap values.

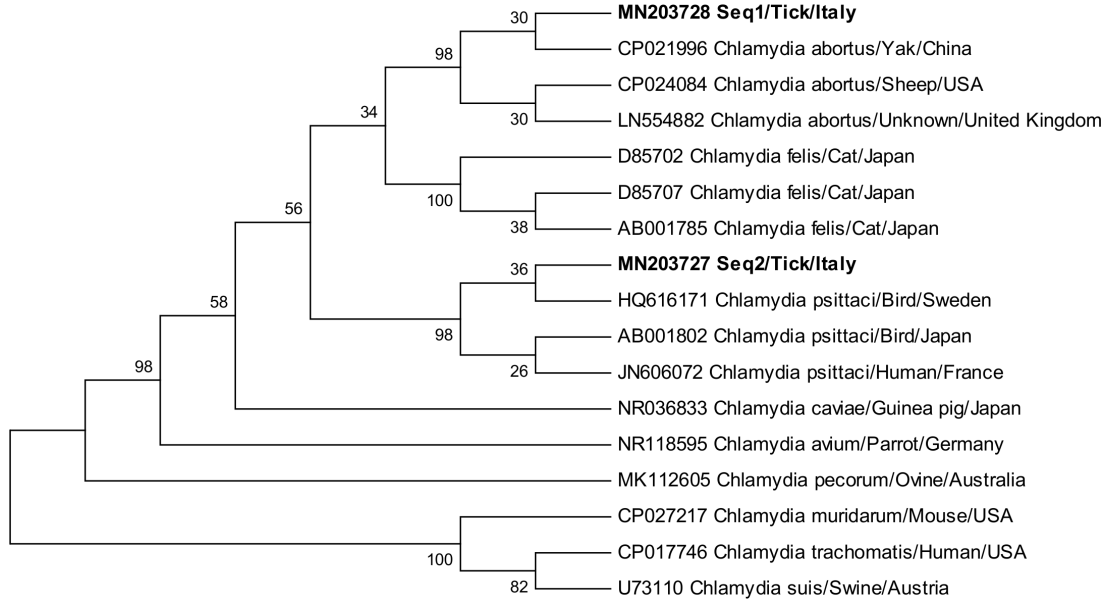


**Figure 2** Molecular phylogenetic analysis by maximum likelihood method based on the Kimura two-parameter model showing the relationship between the Chlamydiales strains obtained from ticks in this study and other nine nucleotide sequences representative of the Chlamydiaceae family. All positions containing gaps and missing data were eliminated. There were a total of 229 positions in the final data set. The numbers at the nodes indicate bootstrap values.

in Tuscany. These ticks have been collected from lizards, mice and hunted wild animals,[28–30] as well as migratory birds, reptiles, domestic animals, people and vegetation in northern and southern Italy.[31–33]

To the best of the authors' knowledge, this is the first report of *B henselae* DNA being detected in *I ricinus* collected from cats and dogs in the locality of Le Grazie. *B henselae*, the causal agent of cat scratch disease, is a zoonotic pathogen transmitted to people through the bites of infected fleas or by scratches from infected cats and dogs—the primary reservoirs of the pathogen.[34 35] Although the cat flea (*Ctenocephalides felis*) is the primary vector of *B henselae*, evidence supports the transmission of *Bartonella* through many different species of ticks. Among them, *I ricinus* has been the focus of studies that support its involvement in *B henselae* transmission.[36 37]

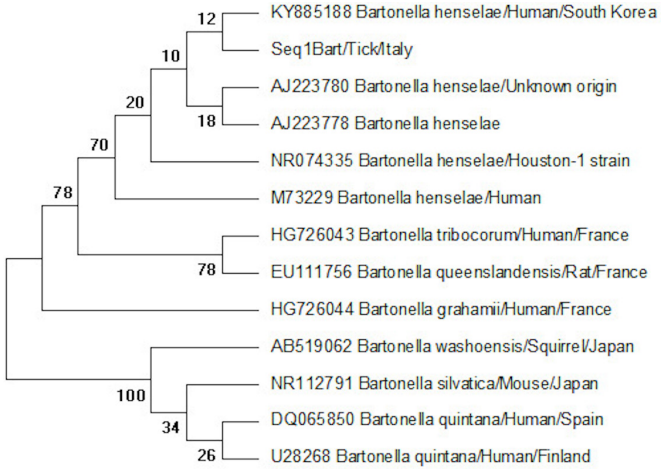


**Figure 3** 16S rRNA-based phylogenetic analyses of the sequence type generated in this study and of 12 sequences representative of the different species of the genus *Bartonella*. In this representative tree, evolutionary history was inferred using the maximum likelihood method based on the Kimura two-parameter model. All positions containing gaps and missing data were eliminated. There were a total of 280 positions in the final data set. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

DNA of different species of *Bartonella* has been detected in *I ricinus* collected from people, wild hosts and vegetation from northern and central Italy.[32 38] In particular, *B henselae* DNA has been detected in *I ricinus* collected from people and vegetation in Italy.[38 39] *B henselae* has also been detected in dogs and cats from central, southern and northern regions of Italy by serological and molecular analyses.[40–45]

As the ticks in this study from which *B henselae* DNA was isolated were partially engorged, the authors cannot establish if the ticks transmitted the *B henselae* DNA to the pets or vice versa. As such, the vector competence of *I ricinus* for *B henselae* transmission cannot be determined. In addition, even though there is currently no evidence that ticks can transmit *Bartonella* to people through bites, the detection of *B henselae* in *I ricinus* indicates that preventive measures should be taken to reduce the risk of possible *B henselae* transmission to people.

This study also reports the detection of *R helvetica* DNA in *I ricinus* collected from a cat. This emerging human pathogen belongs to the spotted fever group *Rickettsiae*. *I ricinus* is the primary vector and reservoir of *R helvetica*,[46] and it has been documented in *I ricinus* ticks collected from people, vegetation, and domestic and wild animal hosts from northern, central and southern Italy.[8 10 47–51] However, there has been some debate on whether *R*

*helvetica* can be transmitted from the ticks to people and animals.

A mild form of *rickettsiosis* serologically attributed to *R helvetica* was found in people in northern Italy.[51] However, the clinical importance of *R helvetica* in domestic animals, as well as whether dogs and cats can serve as a reservoir, is still unknown.[52] A study conducted in Italy highlighted that *R helvetica* was frequently detected in the tick population but not in canine blood samples.[52] However, the serological prevalence of *R helvetica* was determined in cats and dogs in Sweden.[53] The detection of *R helvetica* DNA in *I ricinus* collected from a cat indicates that pets could play a role in spreading infected ticks in the domestic environment.

Based on phylogenetic reconstruction following the maximum likelihood method, the Chlamydiales 16S rRNA genotypes identified in this study were associated with the Chlamydiaceae family, confirming the phylogenetic positioning of these two genetically distinct sequences. The phylogenetic analysis of the two 16S rRNA sequences against other sequences representative of species belonging to the family Chlamydiaceae suggests that the detected sequences unequivocally clustered with *C abortus* and *C psittaci* strains.

This study reports the first detection of *C abortus* and *C psittaci* in *I ricinus* collected from cats. *C abortus* is a pathogen mainly of veterinary importance but also represents a zoonotic risk to people since it can colonise the human placenta and lead to fetal death and miscarriage.[54–56] *C psittaci* strains have been recognised as causes of zoonotic infections such as avian chlamydiosis, epizootic outbreaks in mammals and psittacosis.[55] The occurrence of *C psittaci* infection in dogs and two children that lived with the infected dogs in Germany illustrated the zoonotic potential of the pathogen.[57]

The topic of other potential routes of Chlamydiales transmission, including the use of vectors, has been debated in recent years.[58 59] Evidence exists to support the idea that ticks could be involved in the transmission of Chlamydiaceae, representing a potential source of infection for animals and people. Currently, there is no information about the presence of canine *C abortus* in Italian dogs. This is probably because the pathogen is rarely considered to be a cause of disease in these animals.[54]

In this study, it cannot be known if the pathogens detected in cats and their parasitising ticks shared the same genus within the Chlamydiaceae family. Further studies are needed to understand the role of ticks as a route of transmission for Chlamydiaceae species in people and animals. Since the ticks were collected from the hosts, no conclusions can be drawn regarding the circulation of *C psittaci* and *C abortus* within the *I ricinus* population as every detection can be the result of the ingestion of an infected blood meal.

In this preliminary study, the limited number of ticks collected from a very restricted geographical area does not allow estimation of the incidence of the zoonotic TBDs in this area. However, the detection of *Bartonella*, *Rickettsia* and *Chlamydia* species in *I ricinus* indicates that pets could present a risk to people by increasing the spread of potentially zoonotic pathogens carried by ticks. Further studies are needed to evaluate the role of companion animals in the transmission cycles of tick-borne pathogens.

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