Direct detection of tick-borne pathogens in Ixodes ricinus ticks collected from dogs and cats in Tuscany, Italy

Valentina Chisu (valentinachisu@virgilio.it)
Istituto Zooprofilattico Sperimentale della Sardegna G Pegreffi

Cipriano Foxi
Istituto Zooprofilattico Sperimentale della Sardegna G Pegreffi

Gabriella Masu
Istituto Zooprofilattico Sperimentale della Sardegna G Pegreffi

Barbara D'Amaddio
Istituto Zooprofilattico Sperimentale della Sardegna G Pegreffi

Giovanna Masala
Istituto Zooprofilattico Sperimentale della Sardegna G Pegreffi

Research article

Keywords: Ticks; tick-borne pathogens; Ixodes ricinus; Rickettsia spp.; Chlamydia spp.; Bartonella spp.

Posted Date: September 16th, 2019

DOI: https://doi.org/10.21203/rs.2.14517/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Ticks are able to transmit pathogens which represent an health risk for domestic animals and humans. In this study, ticks morphologically identified as Ixodes ricinus were collected from dogs and cats presented at local veterinary practice in Pistoia, Tuscany. Ticks were examined by PCR in order to detect tick-borne pathogens. Out of 37 I. ricinus ticks analyzed, one tick (3%) was 100% similar to R. helvetica after PCR analyses and sequencing. DNA of Eh. canis was detected in four I. ricinus ticks (12%) . Bartonella spp. DNAs with 100% identity with Bartonella henselae were identified in two I. ricinus (5%) collected from one dog and one cat. One tick showed 100% similarity with C. abortus , while 16 I. ricinus ticks exhibithed DNA of C. psittaci . DNAs of piroplasmids, Anaplasma spp., and C. burnetii were not detected in this study. Findings expand knowledge about the repertoire of tick-borne bacteria present in ticks from Tuscany and indicate that cats and dogs are exposed to tick-borne pathogen which represent a medical risk for their owners.

Introduction

Ticks transmit and carry a large number of pathogens such as bacteria, viruses, protozoan, which have a high impact in human and animal health (Dantas-Torres et al., 2012). In recent years the range of tick-borne diseases affecting domestic animals and humans is increased dramatically, gaining more attention from physicians and veterinarians (Wikel, 2018). Furthermore, ticks are able to parasitize a wide range of animals, including humans that are continuously exposed to zoonotic tick-borne diseases. As consequence of urbanization, the wildlife composition and the related tick population is dramatically changed (Skotarczak, 2018) and humans usually frequent tick-infested areas where both domestic and wild animals live (Jaenson et al., 2009). The companion animals and their associated ticks could concur to the epidemiology of tick-borne diseases in humans, contributing to the tick circulation and to tick-related pathogens in urban area. Therefore, infected ticks carried by these hosts could become a risk not only for the owners but also for the persons that live with the infested animals and that are accidental hosts for several tick species (Skotarczak, 2018).

The Italian tick fauna is one of the most diverse across Europe (Otranto et al., 2014) and is being represented by over 40 species (Manilla, 1988). Among them, Ixodes ricinus represents the most common tick vector of pathogens in central and northern Italy and in all Italian woodland areas where the conditions of humidity and temperature are optimal for its development (Capelli et al., 2012; Maioli et al., 2008; Genchi and Manfredi, 1999). I. ricinus ticks have been also detected in urban and periurban areas (Scarpulla et al., 2019). Dogs and cats that live close to their owners and share the same areas could contribute to the tick spread and vector-borne pathogens in urban area. The objective of the present study was to examine the presence of tick-borne pathogens in ticks collected from dogs and cats presented at their local veterinary practice in Pistoia, Tuscany.

Material And Methods
In September 2018, ticks were collected from two dogs and fifteen cats from a single locality named Le Grazie (close to Pistoia, Tuscany) which is located at 43.93 latitude and 10.92 longitude and at elevation of 63 meters above sea level. All the animals do not presented clinical signs through the veterinary examination. Ticks were removed from hosts with tweezers and placed in vials with 70% of ethanol at room temperature and then delivered to the Experimental Zooprofiliattic Institute of the Sardinia in Sassari, Italy. In the labs of entomology, ticks were morphologically identified and classified in family, genus, and species by using available taxonomic keys and morphometric tables (Manilla, 1998).

**DNA extraction**

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

**Detection and characterization of tick borne pathogens**

Tick DNAs were screened for the presence of *Rickettsia spp.*, *Anaplasma spp.*, *Ehrlichia canis*, *Coxiella burnetii*, *Bartonella spp.*, *Chlamydia spp.*, and *Babesia/Theileria spp.* by using previously established PCR assays (Chisu et al., 2019, 2018a, 2018b). The assay amplifies: 770 bp fragment of the *gltA* gene of *Rickettsia* spp., 240 bp of the *p30* gene of *E. canis*, 293 bp of the 16S rRNA gene of *Anaplasma* spp., 257 bp of the superoxide dismutase gene of *C. burnetii*, 298 bp of the 16S rRNA gene of *Bartonella* spp., 256 bp of the 16S rRNA gene of *Chlamydia* spp., and 411bp of the *Babesia/Theileria* spp. (Chisu et al., 2019, 2018a, b). A negative control using DNA extracted from uninfected ticks and a positive control were included in each test. PCR cycling conditions for all pathogens were the same as documented in referenced publications. The reactions were performed in automated DNA thermal cyclers (GeneAmp PCR Systems 2400 and 9700; Applied Biosystems, Courtaboeuf, France). PCR products were analyzed by electrophoresis in 1.5% agarose gels stained with SYBR® Safe DNA Gel Stain (Thermofisher) and examined under UV transillumination. PCR products were purified using the QIAquick Spin PCR Purification Kit (Qiagen)

**Sequencing assays**

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

**BLASTn and phylogenetic analyses**

Chromatograms of forward and reverse sequences were edited with Chromas 2.2 (Technelysium, Helensvale, Australia), and then aligned with CLUSTALX (Larkin et al., 2007) in order to assign them to unique sequence types, and checked against the GenBank database with nucleotide blast BLASTN (Altschul et al., 1990). Pairwise/multiple sequence alignments and sequence similarities were calculated using the CLUSTALW (Thompson et al., 1994) and the identity matrix options of Bioedit (Hall, 1999), respectively. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013) by using the Maximum Likelihood Method based on models identified as the best with the same software. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

**Sequence accession numbers**

Representative sequences of *Rickettsia gltA* gene, *Chlamydia* and *Bartonella* 16S rRNA genes were submitted to the NCBI database using the National Center for Biotechnology Information (NCBI; Bethesda, MD) BankIt v3.0 submission tool (http://www3.ncbi.nlm.nih.gov/BankIt/). Accession number are MN203727-MN203728 (*Bartonella* 16S rRNA), MN209226-MN209227 (*Chlamydia* 16S rRNA), MN226407 (*Rickettsia gltA*).

**Results**

**Morphological identification of ticks and tick-borne pathogens detected**

A total of 37 ticks were identified by morphological keys as *Ixodes ricinus* as shown in Table 1.

A total of 20 ticks (54%) were positive for the presence of pathogens (Table 1). One out of 37 *I. ricinus* ticks (3%) contained rickettsial DNA sharing 100% sequence identity with the 750-bp *gltA* fragment of *Ri. helvetica* detected in Russia.

Seventeen ticks (46%) were positive for the presence of Chlamydial DNA. In particular, sixteen sequences shared 100% identity with that of *Ch. abortus* and one sequence showed 100% identity with *Ch. psittaci* strains, identified as the closest match by nBLAST. A total of 2 *I. ricinus* ticks collected from one cat and one dog were positive for the presence of *Bartonella* spp. 16S rRNA gene that shared 100% similarity with
B. henselae strains isolated worldwide. None of the collected ticks were positive for Anaplasma spp., E. canis, C. burnetii and Babesia/Theileria species.

Two ticks resulted co-infected with B.henselae/C. abortus; B.henselae/C. psittaci.

**Phylogenetic analyses**

The phylogenetic study of the partial gltA gene was performed by aligning that 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 with R. helvetica strain belonging to Rickettsiae of the spotted fever group (Fig. 1).

Upon sequencing and ClustalX alignment, 16S rRNA sequences were assigned to two different sequence types: Seq1Chl (derived from 16 sequences from I. ricinus ticks from cats) and Seq2Chl (derived from one I. ricinus tick from one cat). 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 (Fig. 2).

The unique sequence type Seq1Bart obtained from the two Bartonella spp. positive sequences grouped with a reference strain representative of B. henselae (Fig. 3).

**Discussion**

In this study, tick-borne pathogens have been identified in I. ricinus ticks collected from dogs and cats from a locality near to Pistoia, a city located in northern Tuscany (central Italy) characterized by a varied landscape: mountains, flat valley, and hill which are rich in vegetation and frequently used for human recreational activity and walking (Ebani et al., 2015). Tick infestations have been previously reported in these areas where wild animals live and that are used for human activity (Ebani et al., 2016). In this study, I. ricinus ticks were collected from companion animals according to other studies that highlight that these ticks represent the most common species present in Tuscany (Ebani et al., 2015).

To the best of our knowledge, this study represents the first direct detection of B. henselae from I. ricinus ticks collected from a dog and a cat from Tuscany region. Bartonella henselae, the agent of the cat scratch disease (CSD), is a zoonotic infectious disease transmitted to humans through bites and scratches from infected cats and dogs (Álvarez-Fernández et al., 2018). Even if the cat flea Ctenocephalides felis is considered the main vector of Bartonella spp., new potential vectors are suspected of transmitting B. henselae, such as I. ricinus, the most abundant ixodid tick in Europe (Medlock et al., 2013). The role of ticks as competent vectors for Bartonella transmission remains controversial (Regier et al., 2017). In Italy, Bartonella DNAs have been detected from I. ricinus ticks collected from humans and by flagging vegetation from northern Italy (Ebani et al., 2015; Sanogo et al., 2003). Bartonella species such as B. acomydis, B. bacilliformis, B. tribocorum have been also detected
from I. ricinus ticks collected from wild hosts in Tuscany (Ebani et al., 2015). The detection of *B. henselae* in *I. ricinus* ticks requires further study in order to investigate the role of ticks as potential source of infection for persons exposed to tick bites.

This study also reports the detection of *R. helvetica* from *I. ricinus* tick collected from a cat from Pistoia, Italy. *Rickettsia helvetica* is an emerging human pathogen belonging to the spotted fever group rickettsiae (SFG). *Ixodes ricinus* ticks have been identified as the main associated vector and reservoir of *R. helvetica* (Portillo et al., 2015). In northern Italy, a mild form of rickettsiosis serologically attributed to *R. helvetica* was found in humans (Fournier et al., 2004). In *I. ricinus* ticks *R. helvetica* has been documented in northeast, central Italy and urban parks (Scarpulla et al., 2019; Mancini et al., 2015; Maioli et al., 2012; Floris et al., 2008; Beninati et al., 2002). Since dogs and cats that live close to humans could play an important role in spreading tick-borne diseases in the peridomestic environment, further research are needed in order to understand if companion animals could play a role as reservoir of *R. helvetica*.

This study reports for the first time the presence of *C. abortus* and *C. psittaci* in *I. ricinus* ticks from Tuscany. *Chlamydia abortus* is mainly of veterinary importance but represents also a zoonotic risk to humans since can colonize the human placenta and lead to foetal death and miscarriage (Essig et al. 2015; Opota et al. 2015; Baud and Greub 2011). *Chlamydia psittaci* strains have been recognized as causes of zoonotic infections such as avian chlamydiosis, epizootic outbreaks in mammals, and psittacosis (Opota et al., 2015). These results support the argument that ticks could be involved in the transmission of *Chlamydiaceae* organisms and represent a potential source of infection for persons exposed to tick bites.

This report increases the repertoire of bacterial pathogens in ticks collected from companion animals and allows to better understand the risk of diseases transmitted by ticks in Tuscany region.

**Declaration**

*Conflict of interest*: The Authors declare that they have no conflict of interest.

**References**

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410

Álvarez-Fernández A, Breitschwerdt EB, Solano-Gallego L (2018) Bartonella infections in cats and dogs including zoonotic aspects. Parasit Vectors. 11:624.

Baud D, Greub G (2011) Intracellular bacteria and adverse pregnancy outcomes. Clinical 240 Microbiol and Infection 17: 1312–1322.
Beninati T, Lo N, Noda H, Esposito F, Rizzoli A, Favia G, Genchi C (2002). First detection of spotted fever group rickettsiae in Ixodes ricinus from Italy. Emerg. Infect Dis 8: 983–986.

Capelli G, Ravagnan S, Montarsi F, Ciocchetta S, Cazzin S, Porcellato E, Babiker AM, Cassini R, Salviato A, Cattoli G, Otranto D (2012) Occurrence and identification of risk areas of Ixodes ricinus-borne pathogens: a cost-effectiveness analysis in north-eastern Italy. Parasit Vectors 5:61.

Chisu V, Alberti A, Zobba R, Foxi C, Masala G (2019) Molecular characterization and phylogenetic analysis of Babesia and Theileria spp. in ticks from domestic and wild hosts in Sardinia. Acta Trop 196:60–65.

Chisu V, Foxi C, Mannu R, Satta G, Masala G (2018a) A five-year survey of tick species and identification of tick-borne bacteria in Sardinia, Italy.Ticks Tick Borne Dis 9:678–681.

Chisu V, Foxi C, Tanda A, Masala G (2018b) Molecular evidence of Chlamydiales in ticks from wild and domestic hosts in Sardinia, Italy. Parasitol Res 117:981–987.

Dantas-Torres F, Chomel BB, Otranto D (2012) Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol 28:437–446.

Ebani VV, Bertelloni F, Turchi B, Filogari D, Cerri D (2015) Molecular survey of tick-borne pathogens in Ixodid ticks collected from hunted wild animals in Tuscany, Italy. Asian Pac J Trop Med 8:714–717.

Ebani VV, Rocchigiani G, Bertelloni F, Nardoni S, Leoni A, Nicoloso S, Mancianti F. Molecular survey on the presence of zoonotic arthropod-borne pathogens in wild red deer (Cervus elaphus). Comp Immunol Microbiol Infect Dis (2016) 47:77–80.

Essig A, Longbottom D (2015) Chlamydia abortus: new aspects of infectious abortion in sheep 279 and potential risk for pregnant women. Curr Clin Microbiol Rpt 2:22–34.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evol 39: 783–791.

Floris R, Yurtman AN, Margoni EF, Mignozzi K, Boemo B, Altobelli A, Cinco M (2008) Detection and identification of Rickettsia species in the northeast of Italy. Vector Borne Zoonotic Dis 8:777–782.

Fournier PE, Allombert C, Supputamongkol Y, Caruso G, Brouqui P, Raoult D (2004) Aneruptive fever associated with antibodies to Rickettsia helvetica in Europe and Thailand. J Clin Microbiol 42: 816–818.

Genchi C, Manfredi MT1999)Tick species infesting ruminants in Italy: ecological and bio-climatic factors affecting the different regional distribution. Parassitologia. 41:41–45.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT Nucleic Acids Symp Ser (Oxford) 41:95–98.
Jaenson TG, Eisen L, Comstedt P, Mejlon HA, Lindgren E, Bergström S, Olsen B (2009) Risk indicators for the tick *Ixodes ricinus* and *Borrelia burgdorferi* sensu lato in Sweden. Med Vet Entomol 23:226–237.

Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol16:111–120.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

Maioli G, Pistone D, Bonilauri P, Pajoro M, Barbieri I, Mulatto P, Vicari N, Dottori M (2012) Etiological agents of rickettsiosis and anaplasmosis in ticks collected in Emilia-Romagna region (Italy) during 2008 and 2009. Exp Appl Acarol 57:199–208.

Mancini F, Ciccozzi M, Lo Presti A, Cell a E, Giovanetti M, Di Luca M, Toma L, Bianchi R, Khoury C, Rezza G, Ciervo A (2015) Characterization of spotted fever group Rickettsia in ticks from a city park of Rome, Italy. Ann Ist Super Sanità 51: 284–290.

Manilla G (1998) Acari Ixodidae, Fauna d’Italia. E Dizioni Calderini, Bologna Medlock JM, Hansford KM, Bormane A, Derdakova M, Estrada-Peña A, George J-C, et al. (2013) Driving forces for changes in geographical distribution of *Ixodes ricinus* ticks in Europe. Parasit Vectors 6:1.

Opota O, Jaton K, Branley J, Vanrompay D, Erard V, Borel N, Longbottom D, Greub G (2015) Improving the molecular diagnosis of Chlamydia psittaci and Chlamydia abortus infection with a species-specific duplex real-time PCR. J Med Microbiol 64: 1174–1185.

Otranto D, Dantas-Torres F, Giannelli A, Latrofa MS, Cascio A, Cazzin S, Ravagnan S, Montarsi F, Zanzani SA, Manfredi MT, Capelli G (2014) Ticks infesting humans in Italy and associated pathogens. Parasit Vectors 14:328.

Portillo A, Santibáñez S, García-Álvarez L, Palomar AM, Oteo JA (2015) Rickettsioses in Europe. Microbes Infect 17:834–838.

Regier Y, Ballhorn W, Kempf VA (2017) Molecular detection of *Bartonella henselae* in *Ixodes ricinus* ticks extracted from a single cat. Parasit Vectors 10:105.

Sanogo YO, Zeaiter Z, Caruso G, Merola F, Shpynov S, Brouqui P, Raoult D (2003) *Bartonella henselae* in *Ixodes ricinus* ticks (Acari: Ixodida) removed from humans, Belluno province, Italy. Emerg Infect Dis 9: 329–332.

Scarpulla M, Barlozzari G, Salvato L, De Liberato C, Lorenzetti R, Macrì G (2018) *Rickettsia helvetica* in Human-Parasitizing and Free-Living *Ixodes ricinus* from Urban and Wild Green Areas in the Metropolitan City of Rome, Italy. Vector Borne Zoonotic Dis 18:404–407.
Skotarczak B (2018) The role of companion animals in the environmental circulation of tick-borne bacterial pathogens. Ann Agric Environ Med 25:473–480.

Tamura K (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution* 9:678–687.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 60. Mol Biol Evol 30: 2725–2729

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Wikel SK (2018) Ticks and Tick-Borne Infections: Complex Ecology, Agents, and Host Interactions. Vet Sci 20:5.

**Table 1**

Table 1. Number of tick species analyzed, hosts, positive ticks tested for pathogens by PCR assays.

| Tick species (number) | Host | *Rickettsia* spp | *Anaplasma* spp. | *E. canis* | *C. burnetii* | *Bartonella* spp. | *Babesia/Theileria* spp. | *Chlamydia* spp. |
|-----------------------|------|------------------|------------------|-------------|---------------|-------------------|------------------------|----------------|
| *I. ricinus* (37)     | Cat  | 1                | -                | -           | 1             | -                 | -                      | 15           |
|                       | Dog  | -                | -                | -           | 1             | -                 | -                      | -            |

**Figures**
Figure 1

Phylogenetic tree of R. helvetica detected in ticks from Pistoia based on partial gltA sequence gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2472)). 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 dataset. The numbers at the nodes indicate bootstrap values.
Figure 2

Molecular Phylogenetic analysis by Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980) showing the relationship between Chlamydiales strain obtained from ticks in this study and other nine nucleotide sequences representative of Chlamydiaceae family. All positions containing gaps and missing data were eliminated. There were a total of 229 positions in the final dataset. The numbers at the nodes indicate bootstrap values.
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 2-parameter model. All positions containing gaps and missing data were eliminated. There were a total of 280 positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches.

Figure 3