Spotted fever group Rickettsiae in *Dermacentor marginatus* from wild boars in Italy

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Abstract

Following the increase in wild boar population recorded in urban and peri-urban areas through Europe, the present survey aimed to assess the occurrence of zoonotic tick-borne bacteria in animals and their ticks collected from southern Italy, in order to evaluate the potential risk of infection for animals and humans. From October to December 2019, a total of 176 ticks collected from 93 wild boars and their spleen samples were molecularly screened for *Borrelia burgdorferi* sensu lato complex, *Coxiella burnetii* and spotted fever group (SFG) *Rickettsia* species. Overall, all the wild boars were infested by ticks (mean intensity, 1.9) with *Dermacentor marginatus* and *Ixodes ricinus* being identified in 99.4% and 0.6%, respectively. Out of 93 wild boars, 17 (18.3%) were infested by ticks positive to spotted fever group (SFG) *Rickettsia* species. *Rickettsia slovaca* and *Rickettsia raoultii* were identified in 16 (9%) and 1 (0.6%) *D. marginatus*, respectively, whereas a single *I. ricinus* (0.6%) was infected by *R. slovaca*. A single wild boar (1.1%) scored positive to *R. slovaca*. All ticks and wild boars scored negative to *C. burnetii* and *B. burgdorferi* s.l. complex. Data herein obtained suggest wild boars are involved in the dissemination of *D. marginatus*, especially in peri-urban settlements of the study area. An integrated management approach is advocated for wild boar population control and preventing the potential risk of tick-borne bacteria in animals and humans.

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Running Title: SFG Rickettsiae in *D. marginatus* of wild boars

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Summary

Following the increase in wild boar population recorded in urban and peri-urban areas through Europe, the present survey aimed to assess the occurrence of zoonotic tick-borne bacteria in animals and their ticks collected from southern Italy, in order to evaluate the potential risk of infection for animals and humans. From October to December 2019, a total of 176 ticks collected from 93 wild boars and their spleen samples were molecularly screened for *Borrelia burgdorferi* sensu lato complex, *Coxiella burnetii* and spotted fever group (SFG)*Rickettsia* species. Overall, all the wild boars were infested by ticks (mean intensity, 1.9) with *Dermacentor marginatus* and*Ixodes ricinus* being identified in 99.4% and 0.6%, respectively. Out of 93 wild boars, 17 (18.3%) were infested by ticks positive to spotted fever group (SFG) *Rickettsia* species.*Rickettsia slovaca* and *Rickettsia raoultii* were identified in 16 (9%) and 1 (0.6%) *D. marginatus* , respectively, whereas a single *I. ricinus* (0.6%) was infected by *R. slovaca* . A single wild boar (1.1%) scored positive to *R. slovaca* . All ticks and wild boars scored negative to *C. burnetii* and*B. burgdorferi* s.l. complex. Data herein obtained suggest wild boars are involved in the dissemination of *D. marginatus* , especially in peri-urban settlements of the study area. An integrated management approach is advocated for wild boar population control and preventing the potential risk of tick-borne bacteria in animals and humans.

Keywords: *Borrelia burgdorferi* , *Coxella burnetii* ,*Dermacentor marginatus* , Italy, SFG *Rickettsia* , wild boar.

Introduction

Tick-borne diseases (TBDs) are of increasing concern in public health worldwide (Otranto et al., 2015). Several socio-demographic factors, as agricultural strategies and wildlife management, deforestation and global warming are strongly involved in the transformation of ecosystems, affecting the tick-host interaction and circulation of tick-borne pathogens (TBPs) (Dantas-Torres et al., 2013; Estrada-Peña and de la Fuente, 2014). Due to the restriction of natural habitats, urbanization may seriously change the composition of wildlife communities and their associated tick populations (Faeth et al., 2011). Human-induced environmental changes may inadvertently select for synanthropic wild mammals, driving the rise of emerging infectious diseases (EIDs) (McFarlane et al., 2012). This could be the case of the wild boars (*Sus scrofa*) which have increased their density throughout Europe, with high occurrence in several countries, including Italy (Pittiglio et al., 2018). In urban and peri-urban areas, the abundance of this mammal may seriously contribute to the maintenance of ticks in the environment (Rizzoli et al., 2014) leading to a high risk of human exposure to them and the pathogens they transmit (Pfäffle et al., 2013).

In the Mediterranean basin, the thermophilic sheep tick*Dermacentor marginatus* (Sulzer, 1776) parasitizes mainly ungulates, being strongly associated to wild boar populations (Nosek, 1972; Di Domenico et al., 2016; Selmi et al., 2017). In addition, adults of *D. marginatus* may feed on humans (Estrada-Peña and Jongejan, 1999; Otranto et al., 2014), mainly in the scalp region (Parola et al., 2009), potentially transmitting zoonotic Gram-negative bacteria such as*Rickettsia* spp., *Borrelia afzelii* and *Coxiella burnetii* (Rehácek et al., 1991; Spitalská et al. 2012; Hornok et al., 2013). In particular, *D. marginatus* is recognized as competent vector of *Rickettsia conorii* (Nosek et al., 1972),*Rickettsia sibirica* and *Rickettsia slovaca* (Estrada-Peña et al., 2014) with the latter being often diagnosed in human patients from Italy, France, Germany and Portugal (Oteo and Portillo, 2012). Both*R. slovaca* and *Rickettsia raoultii* are the causative agent of tick-borne lymphadenopathy (TIBOLA), which is also called*Dermacentor* -borne necrosis erythema and lymphadenopathy (DEBONEL) (Raoult et al., 2002; Parola et al., 2009). In the Mediterranean basin,*R. slovaca* was molecularly detected in 66.7% of *D. marginatus* specimens recovered from wild boars (Leulmi et al., 2016) and, in Algeria, a prevalence of 5.4% was reported in their spleen (Zeroual et al., 2018). Also, lyme disease (LD) by *Borrelia burgdorferi* sensu lato (s.l.) complex being mainly associated to*Ixodes ricinus* (Durden and Beati, 2014) and different species of rodents and reptiles, which act as the main reservoir hosts (Rizzoli et al., 2014; Mendoza-Roldan et al., 2019; 2020). In addition,*Borrelia afzelii* was molecularly detected in *D. marginatus*collected from shepherd dogs in southern Hungary (Hornok et al., 2013) as well as in wild boars from Czech Republic

(Juricová and Hubálek, 2009), northern Portugal (Faria et al., 2015) and central Italy (Ebani et al., 2017). Again, Q fever caused by *Coxiella burnetii,* an obligate *Rickettsia* -related intracellular microrganism affecting several wild and domestic mammal species (Guatteo et al., 2011) was detected in boar populations from different urbanized areas of Germary (Henning et al., 2015) Spain (Toledo et al., 2009) and central Italy (Di Domenico et al., 2016) with prevalence ranging from 1.9% to 8% (Henning et al., 2015; Di Domenico et al., 2016). The detection of*C. burnetii* genotypes infecting humans as well as wild boars from Spain suggested the potential role of this animal species as reservoir of this pathogen (Jado et al., 2012).

The favourable climatic and environmental conditions to the development of different tick species in southern Italy (Dantas-Torres and Otranto, 2013), combined with the increased population density of wild boar in urban and peri-urban areas (Pittiglio et al., 2018) may represent a risk for circulation of TBPs. Based on the paucity of data available on the role of these ungulates in the circulation of tick and pathogens they transmit, this survey aimed to assess the occurrence of zoonotic bacteria in wild boars and their ticks in southern Italy.

Materials and Methods

2.1 Study area and sampling

From October to December 2019, ticks and spleen samples were collected from wild boar's carcasses in different geographical areas of Campania region (southern Italy), under the frame of a wildlife health-monitoring plan (i.e., Boar Emergency Plan in Campania region; authorization no. Decreto Dirigenziale no. 210 - Piano B7 DPAR 2018). For each animal age, gender and location were recorded. Tick samples (n=176) collected from wild boars (n=93) were stored into tubes containing ethyl alcohol (70%) and spleen samples frozen at -20° C. All samples were delivered to the Unit of Parasitology at the Department of Veterinary Medicine, University of Bari "Aldo Moro", Italy. Ticks were morphologically identified and molecularly tested for pathogen DNA as well as spleen specimens. All carcasses were treated in accordance with the rules of the ethic committee of the Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici, Italy) and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2 Morphological identification of ticks

All ticks were classified according to gender (male and female) and developmental stage (larval, nymph, adult) and their feeding status (fed and unfed) was assessed. Tick species were identified by stereomicroscopy (Leica MS5 - Leica Microsystems Ltd. Heerbrugg, Germany) using the morphological keys proposed by Estrada-Peña et al. (2014).

2.3 DNA extraction, PCR protocols and sequencing

DNA was extracted from individual ticks (n=176) and wild boar spleen samples (n=93) using a commercial kit (QIAampDNA Blood & Tissue, Qiagen, Hilden, Germany), according to the manufacturer's instructions. All tick and spleen samples were tested for DNAs of *Borrelia burgdorferi* sensu lato, *Coxiella burnetii* , *Rickettsia*spp. Positive samples to *Rickettsia* spp. were further processed using a specific PCR protocol for SFGR. Details regarding molecular protocols according to the different pathogens investigated in this study are summarized in Table 1. For *Rickettsia* spp., the protocol was modified from Labruna et al. (2004) as follows: 95°C for 10 min initial denaturation, followed by 40 cycles of 95°C for 30s, 58°C for 30s, 72°C for 40s, then 72°C for 7 min for the final elongation. For SFGR, the protocol was modified from Regnery et al. (1991) as follows: 94°C for 10 min initial denaturation, followed by 35 cycles of 94°C for 40s, 58°C for 30s, 72°C for 45s, then 72°C for 10 min for the final elongation. All PCR products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milan, Italy) and visualised on a GelLogic 100 gel documentation system (Kodak, New York, USA). Amplicons were then purified and sequenced in both directions using the same primers as for PCRs by the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic Analyzer (Applied Bio-systems, Foster City, CA, USA). Sequences were edited and analysed by the Geneious software version 9.0 (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012) and compared with those available in the GenBank database by the Basic Local Alignment Search Tool (BLAST;

http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Phylogenetic analysis

The phylogenetic analysis was based on 469bp *omp* A gene sequences of *Rickettsia* spp. detected in different tick and animal species as well as humans, including available sequences from the GenBank database. Phylogenetic relationship was inferred by the Maximum Likelihood (ML) method based on Tamura 3-parameter model (Tamura, 1992) and Gamma distribution used to model evolutionary rate differences among sites (+G) selected by best-fit model (Nei and Kumar, 2000). Evolutionary analyses were conducted on 8000 bootstrap replications using the MEGA X software (Kumar et al., 2018). Homologous sequence from *Rickettsia australis* was used as outgroup (accession number AF149108).

2.5 Statistical analysis

Exact binomial 95% confidence intervals (CI) were established for proportions. The Chi-squared test was used to assess statistical differences of infection rates among gender, feeding status and province of ticks, as well as according to age, gender and province of wild boars. A value of $P < 0.05$ was considered significant. Statistical analysis were performed by using the online software Epitools - Epidemiological Calculators (Sergeant, 2018). The distribution of ticks sampled and positive to *Rickettsia* spp. associated with the administrative provinces of the study area was determined using ArcGIS (version 10.3, ESRI, Redlands, CA, USA).

Results

All wild boars sampled (n=93) were infested by ticks, with a mean intensity of 1.9 ticks (Standard deviation 0.5). Ticks were morphologically identified as *D. marginatus* (175, 99.4%) and *I. ricinus* (1, 0.6%). All ticks were adults, being 77 (43.8%) males and 99 (56.2%) females. Ticks were collected mainly from the groin (60, 34.1%), followed by legs (46, 26.1%), peri-anal (44, 25%) and tail (26, 14.8%). Overall, 18 ticks positive to *Rickettsia* spp. (10.2%; 95% CI: 6.6-15.6) were further identified for SFGR species. In particular, *R. slovaca* and *R. raoultii* were respectively identified in 16 (9%; 95% CI: 5.7-14.3%) and 1 (0.6; 95% CI: 0.1-3.1%) *D. marginatus* , whereas a single *I. ricinus* (0.6%; 95% CI: 0.1-3.1) was infected by *R. slovaca* . Data on the prevalence of infection according to tick gender, feeding status and provenience are reported in Table 2 and Figure 1; no statistically significant difference for *Rickettsia* spp. infections in the ticks examined was observed. Out of 93 wild boars investigated, 17 (18.3%) harboured at least a tick positive for *Rickettsia* spp. with a single wild boar (1.1%; 95% CI: 0.2-5.8) positive for *R. slovaca* , without harbouring any tick. All ticks and wild boar spleens scored negative for *C. burnetii* and *B. burgdorferi* s.l. complex. Consensus sequences of the *omp* A gene analysed displayed 99-100% nucleotide identity with sequences of *R. slovaca* and *R. raoultii* available in GenBank database. *Omp* A partial sequences of *R. slovaca* from ticks and wild boar clustered with high bootstrap value (i.e., 97%) together with those of *D. marginatus* (i.e., HM161787) from Italy and wild boars (MF379311) from Turkey (Figure 2). *Rickettsia raoultii* sequence from *D. marginatus* clustered with a bootstrap value of 85% together with those of the same tick species (i.e., HM161789787) from Italy and human from Hungary (JQ798904). Sequences obtained of *omp* A gene of *R. slovaca* and *R. raoultii* were deposited in GenBank under the accession numbers XXXXXX and YYYYY (AN will be provided in R1) (http://www.ncbi.nlm.nih.gov).

Discussion

The occurrence of tick-borne bacteria such as *R. slovaca* and *R. raoultii* in *D. marginatus* collected from wild boars in southern Italy suggests this animal species has a potential role in the maintenance of this tick species. Indeed, the finding of *D. marginatus* as the most prevalent tick species detected (i.e., 99.4%) in the examined wild boar population, has already been previously reported in several areas of the Mediterranean basin (northeastern Spain, Ortuño et al., 2006; central Italy, Di Domenico et al., 2016; southern Corsica, Grech-Angelini et al., 2016). Although *I. ricinus* is one of the most abundant tick in Europe and vector of many pathogens, including those of zoonotic concern (Petney et al., 2012; Mendoza-Roldan et al., 2019), *D. marginatus* was frequently identified and screened as positive to *Rickettsia monacensis* ,*R. slovaca* and *R. raoultii* collected from human patients in Italy (Otranto et al., 2014). The higher prevalence of *R. slovaca*

(9%) than *R. raoultii* (0.6%) detected in *D. marginatus* is of relevance considering its high pathogenicity in humans (Parola et al., 2009; El Karkouri et al., 2016; Li et al., 2018). Similar data were reported in a survey from northern Italy, showing 32.1% of this tick species harbouring *R. slovaca* and 1.8% of*R. raoultii* (Selmi et al., 2009), as well as *D. marginatus*as the main carrier of *R. slovaca* (Otranto et al., 2014). These findings suggest that in rural and peri-urban contexts (i.e., wooded landscapes and hunting areas), hikers/wild boar hunters and hunting dogs may be exposed to *D. marginatus* , which is commonly retrieved infesting humans and dogs in southern Italy (Otranto et al., 2014). Accordingly, a seroepidemiological survey conducted in Brazil reports that 14.1% of hunting dogs and 14.7% of hunters were exposed to at least one *Rickettsia* species (Kmetiuk et al., 2019). Again, the period in which the incidence of *R. slovaca* infection in humans is higher (Raoult et al., 2002; Parola et al. 2009) overlaps the boar hunting season (i.e., October to December), suggesting a direct relationship between the risk of *Rickettsia* spp. infection and exposure to tickbites. In addition, considering the number of wild boars harbouring at least a tick infected by *Rickettsia* spp. (18.3%), combined to their daily movement capacities (i.e., up to 16 kilometers, Lemel et al., 2003), these ungulates may spread *D. marginatus* and their related pathogens for long distances. This could explain the fact that *Rickettsia* spp. was evenly distributed within the provinces in the study area ($\chi 2$=4.85; P=0.180).

Moreover, considering the increased density and extreme adaptability of wild boar population in urban and peri-urban areas (Massei et al., 2015; Pittiglio et al., 2018), a potential role of this ungulate as spreader of ticks may exist also in these environments. Therefore, the risk of pathogen transmission by this tick species may occur for other hosts, such as foxes (Lorusso et al., 2011), dogs (Maurelli et al., 2018) and humans (Otranto et al., 2014). In addition, the phylogenetic analysis of*omp* A sequences amplified in this study revealed that *R. raoultii* in *D. marginatus* from Italy is closely related to that reported in humans from Hungary, posing a relevant concern for public health. Whereas, *R. slovaca* sequences herein detected, showed a high similarity to those identified in ticks and wild boars from other Mediterranean countries (i.e., Portugal, Turkey), confirming the circulation of this pathogen in the Mediterranean basin.

In this scenario, the risk of infection for humans could be high, considering the lack of knowledge of TBDs and the scant use of protective measures against ticks. Spotted fever is considered an emerging disease in Europe (Portillo et al., 2015) with an increase in clinical case notification in Italy from 1200 (during 1992-1998; Ministero della Sanità, 2000) to 4604 (during 1998-2002; Ciceroni et al., 2006). Recently a decline in the annual incidence of clinical cases (n=5989 from 2001 to 2015) was recorded with a higher occurrence in southern and insular regions (Graziani et al., 2016; Gomez-Barroso et al., 2019). Although the role of wild boars in the epidemiology of SFGR is not completely clarified, our data suggest these animals as involved in the maintenance and spreading of *D. marginatus* and their related SFGR (Selmi et al., 2009; Raele et al., 2018). Although, studies from Italy (Di Domenico et al., 2016) and Spain (Ortuño et al., 2007) would suggest wild boar as candidate reservoir for *R. slovaca* , the detection of pathogen DNA in a single boar which not harboured any infected ticks does not allow any conclusion. In this study, the absence of*Borrelia burgdorferi* s.l. and *C. burnetii* DNA in the ticks and wild boars examined may be explained by the presence of a protein (named defensin) in *D. marginatus* with anti-bacterial activity (Chrudimská et al., 2014). Indeed, other studies have not reported the occurrence of *B. burgdorferi* s.l. in *this* tick, nor in wild boars, in Italy (Martello et al., 2019; Millet et al., 2019) and other European countries (France - Bonnet et al., 2013; Serbia - Tomanovic´ et al., 2013; Romania - Briciu et al., 2014; Spain - Lledó et al., 2014; Portugal - Pereira et al., 2016; Czech Repubblic - Hodžić et al., 2017). Therefore, since few data on the presence of this pathogen in *D. marginatus* is available and considering the role of*I. ricinus* as competent vector and of rodents and reptiles as main reservoirs (Mendoza-Roldan et al., 2019), a negligible involvement of this tick species in the circulation of *B. burgdorferi* s.l. may be hypothesized (Rizzoli et al., 2014). Although the presence of*C. burnetii* in ticks and wild animals from Italy is sporadic (Di Domenico et al., 2016; Ebani et al., 2017), data herein obtained suggest that wild boars and *D. marginatus* may not play a significant role in the circulation of this pathogen in the studied area.

The occurrence of SFGR such as *R. slovaca* and *R. raoultii* , in ticks and wild boars suggest a potential role of these ungulates in the maintenance and spreading of *D. marginatus* in urban and peri-urban areas and suggest that further large-scale investigations are needed to clarify the potential zoonotic source represented

by this ungulate and their ticks.

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Conflicts of Interest:

The authors declare no conflict of interest.

Ethical Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to the European Directive 2010/63/EU, in accordance with the rules of the ethic committee of the Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici, Italy) and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author.

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Table 1

Targeted pathogens and PCR protocols used in this study.

Tick-borne pathogens	**Target gene**
Borrelia burgdorferi sensu lato *Coxiella burnetii Rickettsia* spp. Spotted Fever Group Rickettsiae	Flagellin IS1111a *glt*A

Table 2

Ticks (n=176) infected by *Rickettsia* spp. according to gender, feeding status and province in the studied area with 95% of Confidence Interval (95% CI).

Variables
Gender male female Chi-squared; P-value **Feeding status** (females n=99) fed unfed Chi-squared; P-value **Province** Avel Caserta (CE) Salerno (SA) Chi-squared; P-value

Figure legends

Figure 1. Map showing the distribution of ticks sampled and positive to *Rickettsia* spp. in the study area.

Figure 2. Phylogenetic relationship of *Rickettsia slovaca* and *Rickettsia raoultii* detected in this study to other*Rickettsia* spp. based on a partial sequence of the *omp* A gene. Sequences are presented by GenBank accession number, host species and country of origin.