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Molecular detection and characterization of spotted fever group rickettsiae in ticks from Central Italy

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ABSTRACT

The aim of this study was to investigate the presence of rickettsial pathogens in ticks from Central Italy. A total of 113 ticks hailed from Latium and Tuscany regions were identified and tested by PCR to detect *gltA*, *ompA*, *ompB* genes of *Rickettsia*. Positive amplicons were sequenced and identified at species level. Ticks were analyzed individually or in pools. The percentage of positivity for SFG rickettsiae was 12.4%, expressed as minimum infection rate (MIR) assuming that one tick was positive in each positive pool. *Rickettsia aeschlimannii* was detected in *Hyalomma marginatum*, *Rickettsia monacensis* in *Ixodes ricinus* and *Rickettsia massiliae* and *Rickettsia conorii* in *Rhipicephalus sanguineus* sensu lato. These findings confirm the circulation of pathogenic rickettsiae in Latium and Tuscany regions. To our knowledge this is the first report of *R. massiliae* in Latium region.

1. Introduction

Rickettsia species (order Rickettsiales, α-Proteobacteria) are arthropod-associated Gram-negative bacteria that require eukaryotic host cells to replicate (Hackstadt, 1996; Raoult and Roux 1997; Merhej et al., 2014). *Rickettsia* genus includes an expanding number of species varying in antigenic and microbiological characteristics, distribution, ecology, pathogenicity and association with arthropod hosts such as lice, fleas, ticks and mites, as well as non hematophagous hosts (Eremeeva et al., 2006). This genus includes 27 validated species classified into four groups: spotted fever group (SFG), typhus group (TG), *Rickettsia bellii* group and *Rickettsia canadensis* group (Parola et al., 2013; Merhej et al., 2014). Recently, the use of multiple approaches (phenotypic, genotypic and phylogenic) has been proposed to classify rickettsial isolates and to clarify the taxonomy of *Rickettsia* species (Raoult et al., 2005; Eremeeva et al., 2006). Six genes are the most used to define group, genus and species of rickettsial organisms: sequences of the *16S rRNA* (*rrs*) gene; *gltA* gene encoding rickettsial citrate synthase; *ompA*, *ompB* and *sca4* (gene D) genes encoding rickettsial outer membrane proteins A (rOmpA), B (rOmpB) and PS-120 cell surface antigen (sca4) respectively and *17-kDa* gene encoding 17-kDa outer membrane antigen (Anderson and Tzianabos, 1989; Simser et al., 2002; Fournier et al., 2003; Raoult et al., 2005).

SFG rickettsiae have been isolated worldwide and their geographical distribution varies depending on *Rickettsia* species. Usually associated with ixodid ticks, they are transmitted to vertebrates through salivary secretions and maintained in ticks by transovarial transmission or horizontal acquisition after ingestion of infected blood meals (Beninati et al., 2005). Some members of SFG, such as the causative agent of Mediterranean spotted fever (MSF) *Rickettsia conorii* and the agent of Rocky Mountain spotted fever (RMSF) *Rickettsia rickettsii*, are human pathogens responsible for disseminated infections and causative agents of tick-borne eruptive fevers due to their ability to replicate in endothelial cells (Fournier et al., 1998; Merhej and Raoult, 2011; Merhej et al., 2014).

Until 2002 the only SFG *Rickettsia* species reported in Italy was *R. conorii* (Beninati et al., 2005). Since then, an increasing number of species of this genus was detected in ticks and in clinical samples. *R. helvetica* was found in *I. ricinus* and *I. acuminatus* (Beninati et al., 2002; Sanogo et al., 2003; Bertolotti et al., 2006; Floris 2008 et al.; Maioli et al., 2012; Tomassone et al., 2013); *R. conorii* subsp.

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israelensis in *Rh. sanguineus* sensu lato (Sanogo et al., 2003; Chisu et al., 2014); *R. conorii* Indian tick typhus strain in humans (Torina et al., 2012); *Rickettsia slovaca* in *Dermacentor marginatus*, *Haemaphysalis punctata* and humans (Beninati et al., 2005; Selmi et al., 2008; Selmi et al., 2009; Masala et al., 2012; Maioli et al., 2012; Torina et al., 2012); *Rickettsia africae* in *H. marginatum* and *R. aeschlimannii* in *H. marginatum* and *Amblyomma* spp. ticks (Beninati et al., 2005; Mura et al., 2008; Tomassone et al., 2013; Toma et al., 2014); '*Candidatus* Rickettsia barbariae' in *Rh. turanicus* (Mura et al., 2008); *Rickettsia raoultii* in *D. marginatus* (Selmi et al., 2009; Maioli et al., 2012); *Rickettsia limoniae* in *I. ricinus* (Floris et al., 2008) and *Rickettsia monacensis* in *I. ricinus* and in a patient with MSF-like illness (Beninati et al., 2002; Floris et al., 2008; Maioli et al., 2012; Madeddu et al., 2012; Castro et al., 2015). *Rickettsia massiliae* was retrospectively identified in a human patient and was detected in *Rh. turanicus, Rh. sanguineus* s.l. and *I. ricinus* (Vitale et al., 2006; Mura et al., 2008; Trotta et al., 2012; Cascio et al., 2013; Castro et al., 2015).

The aim of this study was to investigate the presence of rickettsial pathogens in ticks from Central Italy by specific SFG *Rickettsia* DNA amplification and sequencing.

2. Materials and methods

2.1. Sampling and identification

A total of 113 ticks hailed from different provinces of Latium and Tuscany regions (Arezzo, Livorno, Massa Carrara, Pistoia and Roma) were investigated. Ticks were sent to Zooprophylaxis and Research Institute of Latium and Tuscany "M. Aleandri" in 2012–2013 and stored in sterile tubes with 70% ethanol at room temperature until identification and DNA extraction. Ticks were morphologically classified into family, genus and species using taxonomic keys (Manilla, 1998; Iori et al., 2005).

2.2. DNA extraction

Ticks were dried on sterile filter paper discs and cut with a scalpel in Petri dishes. Some ticks belonging to the same species and stage, hailed from the same animal and area, were analyzed in pools. Samples were homogenized using FastPrep® by adding 600 μl of phosphate buffer solution (PBS) in Lysing Matrix D tubes (MP Biomedicals, Germany). Tubes were centrifuged at 8000 rpm for 1 min and supernatant was discarded. ATL buffer and Proteinase K were added directly in the Lysing Matrix D tubes. Genomic DNA was extracted using QIAamp DNA mini Kit (Qiagen, Hilden, Germany) according to tissue manufacturer's instructions.

2.3. PCR amplification and electrophoresis

A PCR targeting a 450–500-bp fragment of tick *28S r*RNA gene using the primers 28SF (5′-GAC-TCT-AGT-CTG-ACT-CTG-TG-3′) and 28SR (5′-GCC-ACA-AGC-CAG-TTA-TCC-C-3′) was performed to ensure the effectiveness of the nucleic acid extraction (Inokuma et al., 2003). When DNA was not successfully extracted, a second extraction was performed on the residual pellet previously stored at −20 °C.

The presence of *Rickettsia* spp. DNA was screened through a PCR assay which amplify a 381-bp fragment of the citrate synthase gene (*gltA*) common to *Rickettsia* genus using the primers RpCs877p (5′-ATG-GCG-AAT-ATT-TCT-CCA-AAA-3′) and RpCs1258n (5′-ATT-GCA-AAA-AGT-ACA-GTG-AAC-A-3′) (Regnery et al., 1991). Samples were further analyzed to detect the rickettsial outer-membrane protein genes o*mpA* and *ompB* (Roux and Raoult, 2000).

A *ompA* semi-nested PCR (snPCR) with the primers Rr190.70p (5′-ATG-GCG-AAT-ATT-TCT-CCA-AAA-3′), Rr190.701n (5′-GTT-CCG-TTA-ATG-GCA-GCA-TCT-3′) and Rr190.602n (5′-AGT-GCA-GCA-TTC-GCT-CCC-CCT-3′) was performed to amplify a 532-bp fragment of the *ompA* gene (Oteo et al., 2006). Since *ompA* gene allows to detect SFG rickettsiae except *Rickettsia helvetica*, *Rickettsia akari*, *Rickettsia australis*, and *R. bellii,* a PCR was performed to amplify *ompB* gene (Roux et al., 1996).

A *ompB* nested-PCR (nPCR) was performed to amplify a 420-bp fragment of the *ompB* gene common to all SFG and TG rickettsiae. The first amplification was performed with the primers *ompB*-OF (5′-GTA-ACC-GGA-AGT-AAT-CGT-TTC-GTA-A-3′)/*ompB*-OR(5′-CTT-TAT-AAC-CAG-CTA-AAC-CAC-C-3′), while the second amplification was performed with the primers *ompB* SFG-IF (5′-GTT-TAA-TAC-GTG-CTG-CTA-ACC-AA-3′)/*ompB* SFG/TG-IR (5′-GGT-TTG-GCC-CAT-ATA-CCA-TAA-G-3′) (Choi et al., 2005).

R. conorii DNA extracted from IFA slides (Fuller Laboratories, Fullerton, California, USA) was used as positive control in all PCRs. Nuclease-free water was used as negative control. PCRs were performed in GeneAmp® PCR System 9700—Applied Biosystems DNA thermal cycler; amplification products were visualized by electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, Inc., Hayward, CA) and examined under UV transilluminator.

2.4. DNA sequencing

Amplicons (*gltA*, *ompA*, *ompB*,) were purified by the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA), quantified and sent for sequencing (© 2014 Macrogen Inc., Macrogen Europe, Amsterdam the Netherland). The obtained sequences were aligned using Bioedit software (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) and analyzed for nucleotide sequence identity by comparing them with reference strains in GenBank database using the Basic Local Alignment Search Tool (BLAST).

3. Results

A total of 113 ticks were analyzed. From those, 89 were removed from animals (dog, cat, fox and wolf), 4 from humans and 20 were free-living. Ticks were 101 adults and 12 nymphs, morphologically identified as *Ixodes ricinus* 35.4% (40/113), *Rhipicephalus turanicus* 31.9% (36/113), *Rhipicephalus sanguineus* sensu lato 22.1% (25/113), *Hyalomma marginatum* 8.8% (10/113), *Ixodes acuminatus* 0.9% (1/113) and *Ixodes hexagonus* 0.9% (1/113) (Table 1). Tick species, number, stage, source and collection site for each province are summarized in Table 2. Seventy-five ticks were analyzed in 18 pools of maximum 10 ticks while 38 were individually tested. The percentage of positivity was expressed as minimum infection rate (MIR) assuming that one tick was positive in each positive pool. Minimum infection rate of ticks by *Rickettsia* spp. was 14.2% (16/113) (Table 3) while MIR by SFG rickettsiae was 12.4% (14/113). SFG rickettsiae were detected both in ticks removed from hosts and in free-living ones with a MIR of 12.9% (12/93) and of 10.0% (2/20), respectively. SFG rickettsiae were detected in ticks from cat, dog, human or free-living (Table 2). One (10.0%) *H. marginatum* was positive for *Rickettsia aeschlimannii*, 11 (27.5%) *I. ricinus* were positive for *R. monacensis*, 1 (4.0%) and 1 (4.0%) *Rh. sanguineus* s.l. was posi-

Table 1
Number and percentage of identified tick species.

Tick species (n)	%
Hyalomma marginatum (10)	8.8
Ixodes acuminatus (1)	0.9
Ixodes ricinus (40)	35.4
Ixodes hexagonus (1)	0.9
Rhipicephalus sanguineus sensu lato (25)	22.1
Rhipicephalus turanicus (36)	31.9
Total (113)	100