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Characterization of spotted fever group *Rickettsiae* in ticks from a city park of Rome, Italy

Fabiola Mancini[1], Massimo Ciccozzi[1,2], Alessandra Lo Presti[1], Eleonora Cella[1], Marta Giovanetti[1], Marco Di Luca[1], Luciano Toma[1], Riccardo Bianchi[1], Cristina Khoury[1], Giovanni Rezza[1] and Alessandra Ciervo[1]

[1]*Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Rome, Italy*
[2]*Università Campus-Biomedico, Rome, Italy*

Abstract

Background. Ticks are vectors and important reservoirs for microbial agents that cause disease in humans and animals. Among these pathogens, the members of *Rickettsia* species play an important role in public health.

Aim and methods. One hundred twenty-nine ticks belonging to four tick species (*Ixodes ricinus*, *Rhipicephalus turanicus*, *Dermacentor marginatus*, and *Haemaphysalis punctata*) were collected at different sites of the Insugherata Natural Reserve, localized in the urban area of Rome, Italy. Questing ticks were tested by PCR for *Rickettsia* spp., amplifying partial gene of *omp*A.

Results. Forty-six ticks were found to be infected with *Rickettsia* species. Five SFG *rickettsiae* were identified: three human pathogens *Rickettsia conorii, Rickettsia massiliae* and *Rickettsia aeschlimannii*, and two putative new strains *Rickettsia* sp. strain RM1 and *Rickettsia* sp. strain RM2. The phylogenetic analysis of partial gene sequences of *omp*A, *glt*A, and 17-kd antigen showed that they clustered with several *rickettsiae* with unidentified pathogenicity. However, *Rickettsia* sp. strain RM1 and *Rickettsia* sp. strain RM2 clustered in a statistically supported clade with *R. massiliae,* and *R. monacensis*, respectively.

Conclusion. Our findings suggest that *Rickettsia* species other than *R. conorii* are implicated in human disease in Italy.

Key words

- tick
- urban park
- *Rickettsia* SFG
- molecular characterization

INTRODUCTION

Ticks are known to be vectors and important reservoirs for microbial agents that cause disease in humans and animals. Among these pathogens, the members of *Rickettsia* species play an important role in public health. The genus *Rickettsia* is divided into three groups on the basis of phenotypic criteria: the spotted fever group (SFG), the typhus group (TG), and the scrub typhus group (STG) which is absent in Europe [1].

The most common and well-known tick-borne rickettsiosis in Europe is the Mediterranean Spotted Fever (MSF). The MSF is due to *Rickettsia conorii,* but in the last decade other rickettsial species, such as *R. slovaca*, *R. sibirica mongolotimonae*, *R. helvetica*, *R. monacensis*, *R. massiliae*, *Rickettsia aeschlimannii*, *R. africae* or *R. akari,* were identified in the Mediteranean basin and have been implicated or potentially involved in human diseases [1]. In the past, identification and differentiation of *rickettsiae* were based exclusively on serological data, ecology, and epidemiology of these microorganisms. The discovery of these new species has been made possible by the use of molecular identification techniques. Molecular methods can offer a real burst in the investigation of novel *Rickettsia* species and/or their interaction with new vectors. In particular, PCR based method and DNA sequencing are helpful tools for the identification of rickettsial DNA in a variety of human specimens and arthropods, and allows the simultaneous detection of different microorganisms in the same sample. Monitoring vector distribution and the prevalence of tick-transmitted pathogens is therefore essential to describe and understand the risk of tick-borne disease.

In Italy, from 1998 to 2002, 4604 clinical cases of rickettsiosis with 33 deaths were reported [2]. Italian regions with elevated incidences of rickettsial diseases are Sicily, Sardinia, Lazio, and Calabria, while in other regions, rickettsiosis is sporadic [2].

Currently, in Italy, several studies are focused on molecular identification and characterization of *Rickettsia* spp. in ticks and human samples to verify the potential

Address for correspondence: Alessandra Ciervo, Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: alessandra.ciervo@iss.it.

presence of species that have been recently discovered in other parts of Europe [3-8].

In order to provide a useful contribution in this field, we analyzed the presence of *Rickettsia* spp. in questing ticks collected in an urban park of Rome, during an entomological survey conducted in 2011 [9]. The aims of the present study were i) to investigate the prevalence of *Rickettsia* spp. in ticks collected in the public park of Rome, highly frequented by daily visitors and used for recreational activities; and ii) to characterize *rickettsiae* in infected ticks using molecular methods, including PCR, sequence, and phylogenetic analyses. Hereby we report the results of this investigation.

MATERIALS AND METHODS

Tick collection

Questing ticks were collected in the Insugherata Natural Reserve, localized in the north-western sector of Rome and connected to the green zones outside the urban area. The park is characterized by woods and bush. Moreover, the reserve, with its Mediterranean climate hosts a rich fauna: foxes, weasels, and porcupines are very common, whilebadgers occur only sporadically. Many small mammals (*Apodemus sylvaticus, Microtus savii, Suncus etruscus, Erinaceus europaeus, Talpa europaea, and Muscardinus avellanarius*) and a great variety of birds, reptiles, and amphibians complete the wild fauna of the reserve. Only in the past few years, wild boar have spread from northern boundaries of the park. Tick collections were conducted in three selected sites within the park twice a month from January to December 2011, along transects of 100 m each for a total of 12 fixed transects covered per visit. Questing ticks were collected in all sites by dragging a 1 m^2 woolen blanket through the vegetation [9].

Ticks were identified according to morphological characters [10], and stored at -80 °C.

DNA extraction

Ticks were individually dissected and homogenized under sterile conditions. Genomic DNA was extracted using Dneasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturing protocol. DNA samples were stored at -20 °C and later used as templates for the PCR amplification.

Rickettsiae DNA detection

Detection of *Rickettsia* spp. DNA was done with primers RpCS.877p–RpCS.1258n of the citrate synthase gene (*glt*A) [11]. Two different sets of primers of the *gro*EL gene [12], were used for the discrimination between SFG and TG *(Table 1)*. PCR products were resolved by electrophoresis on a 1.5% agarose gel, then stained with ethidium bromide, and visualized under UV light.

The genomic DNA of *R. conorii* and *R. typhi*, were used as positive controls in specific PCR analyses.

PCR amplification and sequencing of specific rickettsial gene target fragments

As shown in *Table 1*, PCRs were performed using oligonucleotide primers: CS409d and Rp1258n which amplify a 750 bp fragment of the *glt*A [13]: Rr 190.70 and Rr 190.701 for the outer surface protein rOmpA (*omp*A gene), which amplify a 629-632 bp portion [14]; Rr17.13 and Rr17.495r of the17-kDa protein (17-kd gene), which amplify a 400 bp fragment [15].

PCR products were purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and amplicons were sequenced in the forward and reverse directions with the same primer pairs used for the PCR amplifications. Sequencing was performed by Bio-Fab research (Italy; http://www.biofabresearch.it), and DNA sequences were compared with available databases in GenBank using the Basic Local Alignment Search Tool (BLAST) on http://blast.ncbi.nlm.nih.gov.

The rickettsial nucleotide sequences of the partial *glt*A, *omp*A and 17-kd genes were submitted to the NCBI GenBank.

Phylogenetic analysis

Three different datasets were built. The first one included the rickettsial nucleotide sequences of the partial *omp*A gene isolated from Rome, plus 165 representative rickettsial species sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/). The second

Table 1
Primers and probes used for detection of rickettsial pathogens in ticks

Organism	Gene target	Primer/Probe sequence (5'→ 3')
Rickettsia spp.	gtlA	GGGGACCTGCTCACGGCGG ATTGCAAAAAGTACAGTGAACA
Rickettsia (TG and SFG)	groEL	GATAGAAGAAAAGCAATGATG CAGCTATTTGAGATTTAATTTG
Rickettsia (TG)	groEL	GGTGAAGCACTTGCGACG AGGAGCTTTTACTGCTGC
Rickettsia spp.	gtlA	CCTATGGCTATTATGCTTGC ATTGCAAAAAGTACAGTGAACA
Rickettsia spp.	ompA	ATGGCGAATATTTCTCCAAAA GTTCCGTTAATGGCAGCATCT
Rickettsia spp.	17-kd antigen	TAGAGAGAATTATATGAAACTATTATC ATGACGTTTTGTCTATCAATTCAC

dataset included the rickettsial nucleotide sequences of the partial *glt*A gene, isolated from Rome, plus 122 representative rickettsial species sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/). The third dataset included the rickettsial nucleotide sequences of the partial 17-kd gene collected from Rome, plus 76 representative rickettsial species sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/). The sequences of all datasets were aligned using Clustal X software [16], and then manually edited using Bioedit software [17]. Version 3.7 of the ModelTest program was used to select the evolutionary model that best fitted the sequence data [18]. Maximum likelihood phylogenetic trees were constructed with the GTR + I + G nucleotide substitution model for the first dataset, with the HKY + I + G nucleotide substitution model for the second dataset, and with the K80 + I + G nucleotide substitution model for the third dataset.

The phylogenetic signal of each sequence dataset was investigated by means of the likelihood mapping analysis of 10 000 random quartets, generated using TreePuzzle [19].

For a quartet, just three unrooted tree topologies are possible. The likelihood of each topology is estimated with the maximum likelihood method and the three likelihoods are reported as a dot in an equilateral triangle (the likelihood map). Three main areas in the map can be distinguished: the three corners representing fully resolved tree topologies, *i.e.* the presence of treelike phylogenetic signal in the data; the center, which represents star-like phylogeny, and the three areas on the sides indicating network-like phylogeny, *i.e.* presence of recombination or conflicting phylogenetic signals. When using this strategy, if more than 30% of the dots fall into the center of the triangle, the data are considered unreliable for the purposes of phylogenetic inference.

Maximum likelihood phylogenetic trees were constructed with Phyml [20]. Statistical robustness and reliability of the branching order within the phylogenetic trees were confirmed by bootstrap analysis.

RESULTS

Tick collection

A total of 325 questing ticks were collected, and a representative sample, randomly selected, of 129 ticks was processed for *Rickettsia* spp. analyses. *Rhipicephalus turanicus* was the most abundant species (66%) with 29 males and 56 females, followed by *Ixodes ricinus* (26%) with 11 males and 22 females, *Dermacentor marginatus* (5%) with 1 males and 6 females, and *Haemaphysalis punctata* (3%) with 1 males and 3 females. *R. turanicus* showed a seasonal pattern from spring to early summer, while *I. ricinus* and *D. marginatus* resulted active from October to May and from October to April, respectively. *H. punctata* was rare, with a seasonal activity in autumn-winter [9].

Rickettsia spp. detection

Out of the 129 ticks screened through the *glt*A gene target [11], 46 (36%) samples were positive for *Rickettsia* spp. In particular, rickettsial DNA was found in *R. turanicus* (22/85; 26%), *I. ricinus* (23/33; 70%), and *D. marginatus* (1/7; 14%), while any *H. punctata* tick was positive for the presence of the pathogen *(Table 2)*. *I. ricinus* was about 2.5 times more likely to be infected by *Rickettsia* spp. than *R. turanicus*. Specific *gro*EL PCR reactions for TG and SFG discrimination [12], determined that all rickettsiae belonged to SFG.

Rickettsia spp. identification

To determine the diversity of SFG *rickettsiae*, DNA from the 46 positive individual ticks was subjected to partial amplification and sequencing of gene encoding, *ompA* as previously reported [14]. The sequences obtained were compared to other bacterial sequences present in the GenBank database. A total of five different *Rickettsia* SFG species were identified.

As shown in *Table 2*, a 100% identity to the *ompA* fragment sequences was obtained for: *R. monacensis* (*R. monacensis* isolate 3IRF MA, GenBank accession number KF258154), from 13 *I. ricinus*, 7 *R. turanicus*, and 1 *D. marginatus*; *R. massiliae* (*R. massiliae* MTU5,

Table 2
Identification of Rickettsia spp. in tick samples

Tick species	N. of ticks infected/ total n. of ticks examined	Infection rate (%)	*Rickettsia* spp. identified (n.)§	Identity (%) / GenBank accession number§§
R. turanicus	22 (85)	26	*R. monacensis* (7) *Rickettsia* sp. strain TwKm01 (7) *R. massiliae* (6) *R. conorii* (1) *R.. aeschlimannii* (1)	(100) / KF258154 (99) / EF219467 (100) / CP000683 (100) / AE006914 (100) / HQ335158
I. ricinus	23 (33)	70	*R. monacensis* (13) *R. massiliae* (4) *R. aeschlimannii* (3) *R. conorii* (2) *Rickettsia* sp. strain TwKm01 (1)	(100) / KF258154 (100) / CP000683 (100) / HQ335158 (100) / AE006914 (99) / EF219467
D. marginatus	1 (7)	14	*R. monacensis* (1)	(100) / KF258154
H. punctata	0 (4)	0	0	
Total	**46 (129)**	**36**		

§ The identification was established based on the partial gene sequence of *ompA*
§§ The identity was established based on the partial gene sequence of *ompA*

GenBank accession number CP000683), from 6 *R. turanicus,* and 4 *I. ricinus*; *R. conorii* (*R. conorii* strain Malish 7, GenBank accession number AE006914), from 2 *I. ricinus,* and 1 *R. turanicus; R. aeschlimannii* (*R. aeschlimannii* EL-Arish-18, GenBank accession number HQ335158), from 3 *I. ricinus,* and 1 *R. turanicus*. In contrast, a 99% identity to the partial *ompA* sequence of *Rickettsia* sp. strain TwKm01 (GenBank accession number EF219467) was found from 7 *R. turanicus,* and 1 *I. ricinus*. This percentage of identity is due to an insertion (216 nucleotide position) of three consecutive nucleotides (GAT), with an introduction of a putative predicted aspartic aminoacid in the sequence of the rOmpA protein.

Sequences from these eight ticks were identical to one another and, in support of a better characterization, the *gtlA* and the 17-kDa antigen partial gene sequences were also performed and compared in the GenBank [13, 15].

For seven samples, sequence analysis of *gtlA* showed 99% identity to the partial *gtlA* sequence of *Rickettsia* sp. strain TwKm01 (GenBank accession number EF219463), and were named *Rickettsia* sp. strain RM1. All sequences displayed a nucleotide exchange (C→T) at 845 nucleotide position with an aminoacid replacement (His→Tyr) in the gtlA protein sequence. Otherwise, one sample presented a 100% identity to the partial *gtlA* sequence of *Rickettsia* sp. strain IRS4 (GenBank accession number AF141906) and were named *Rickettsia* sp. strain RM2.

Partial sequence analysis to the 17-kDa antigen of the *Rickettsia* sp. strain RM1 showed a 99% identity with sequences of the *Rickettsia* sp. TwKM01 from Taiwan (GenBank accession number AY445821) and *R. rhipicephali* (GenBank accession number U11020), with a nucleotide exchange (G→A) or (T→C) at position 217 or 140 in the nucleotide sequences, respectively. In contrast, the *Rickettsia* sp. strain RM2 displayed a 99% identity with the 17-kDa sequence of the *Rickettsia* sp. 777c (GenBank accession number EU283838) due to a several nucleotide switches (G→A at position 96, A→G at position 157, T→C at position 168, A→G at position 385) in the nucleotide sequence with 2 aminoacids replacement (Met→Ile at position 16, and Ser→Gly at position 37).

Phylogenetic analysis

The phylogenetic noise of each data set was investigated by means of likelihood mapping. The percentage of dots falling in the central area of the triangles was 28% for the first dataset, 11.2% for the second dataset, and 15.6% for the third dataset: as none of the datasets showed more than 30% of noise, all of them contained a sufficient phylogenetic signal *(Figure 1 a, b, c)*.

Maximum likelihood phylogenetic tree of the first dataset (partial *omp*A gene) was shown in *Figure 2*. All *rickettsia* isolates clustered together in a statistically supported cluster, which included the following reference sequences: a sequence *Rickettsia* sp. TwKM01 from Taiwan (GenBank accession number EF219467), two rickettsia sequences from China (*Rickettsia* sp. ZJW4-3/2007, FJ176299; *Rickettsia* sp. ZJ43/2007, EU258735), and three rickettsia sequences from Cyprus (CyRtu43H, JF803899; CyRtu 43D, EU448158; CyRtu 43S, EU448159).

The maximum likelihood phylogenetic tree of the second dataset (partial *glt*A gene) was shown in *Figure 3*. The maximum likelihood analysis identified seven rickettsia isolates (sequence labelled as RM1) in a statisti-

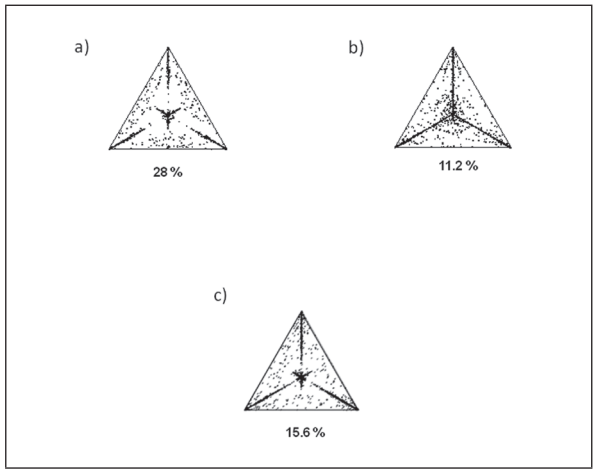

Figure 1
Likelihood mapping of rickettsia sequences using the *gtl*A dataset (a), the *omp*A dataset (b) and the 17-kDa dataset (c). The dots inside the triangles represent the posterior probabilities of the possible unrooted topologies for each quartet. Numbers in the centre of the triangles indicate the percentage of dots in the centre of the triangle corresponding to phylogenetic noise (star-like trees). The figure is also available online as supplementary material in an enlarged version.

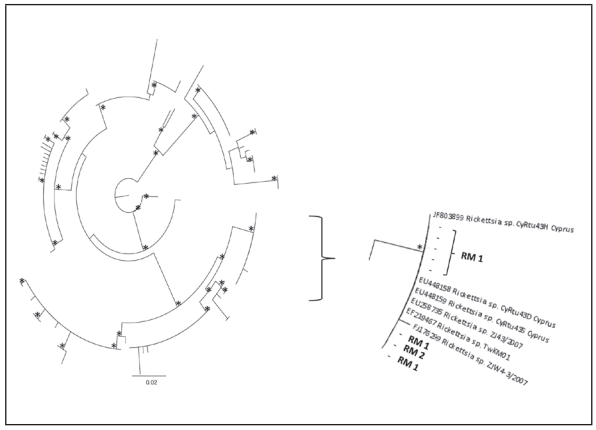

Figure 2
Maximum likelihood phylogenetic analysis of rickettsia partial *omp*A gene sequences (second dataset). Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test and were drawn in scale with the bar at the bottom indicating 0.02 nucleotide substitutions per site. The * along the branch represents significant statistical support for the clade subtending that branch (bootstrap support > 75%). The tree is midpoint rooted. The Italian isolates (collected in Rome) are shown in bold. The figure is also available online as supplementary material in an enlarged version.

cally supported cluster with a strain from Taiwan, *Rickettsia* sp. strain TwKm01 (GenBank accession number EF219463). Moreover, this cluster was included in a statistically supported clade with representative *Rickettsia* sequences such as strains of *R. massiliae* (GenBank accession number: KF826286, HM050293, JN043507, U59719), *Rickettsia* sp. Bar 29 (GenBank accession number U59720), *Rickettsia* sp. PoTiR600 from Portugal (GenBank accession number HM149282), a candidatus *Rickettsia kulagini* strain Kertch (GenBank accession number DQ365806), two strains of *R. rhipicephali* (GenBank accession number: U59721, DQ865206) and *Rickettsia* sp. R300 from Brazil (GenBank accession number AY472038).

One rickettsia isolate (sequence labelled as RM2) was found in a statistically supported clade including reference sequences such as *Rickettsia* sp. IRS4 collected in Slovakia (GenBank accession number AF141906), *Rickettsia* sp. PoTiR6dt (GenBank accession number EF501756) collected in Portugal, *R. monacensis* strain IR/Munich (GenBank accession number DQ100163), *Rickettsia* sp. CH -73-2 from Switzerland (GenBank accession number EU359298), *R. monacensis* from China (GenBank accession number: EU665236, EU665235), *R. monacensis* strain CN45kr from South Korea, *Rickettsia* sp. PoTiR5td from Portugal (GenBank accession number EF501755) and *Rickettsia* sp. IRS3 from Slovakia (GenBank accession number AF140706). Outside of this clade there was a rickettsia reference sequence labelled *Rickettsia* sp. 12G1 (GenBank accession number KF831358) isolated from Ecuador.

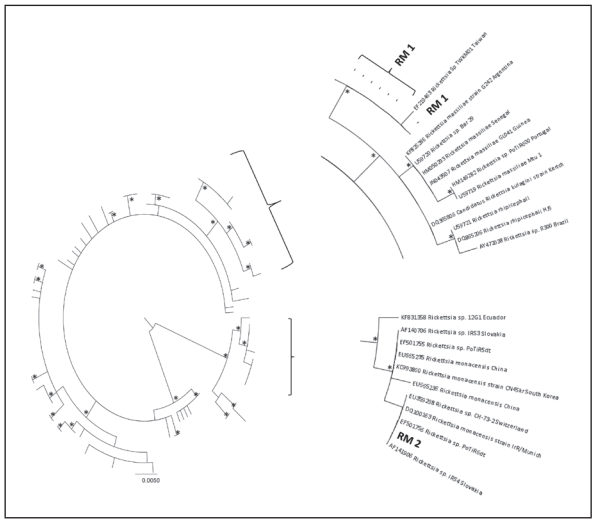

Figure 3
Maximum likelihood phylogenetic analysis of Rickettsia partial *glt*A gene sequences (second dataset). Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test and were drawn in scale with the bar at the bottom indicating 0.0050 nucleotide substitutions per site. The * along the branch represents significant statistical support for the clade subtending that branch (bootstrap support > 75%). The tree is midpoint rooted. The Italian isolates (collected in Rome) are shown in bold. The figure is also available online as supplementary material in an enlarged version.

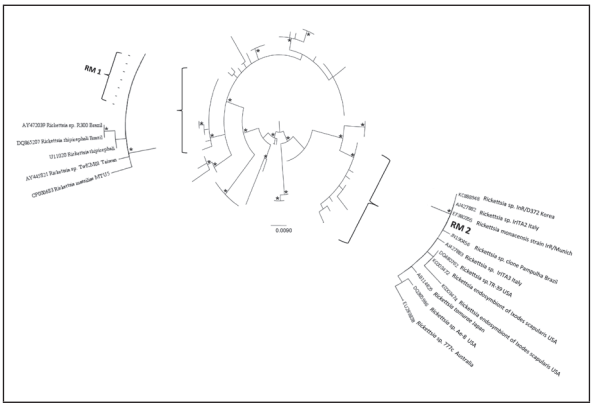

Figure 4
Maximum likelihood phylogenetic analysis of rickettsia partial 17-kDa gene sequences (third dataset). Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test and were drawn in scale with the bar at the bottom indicating 0.0090 nucleotide substitutions per site. The * along the branch represents significant statistical support for the clade subtending that branch (bootstrap support > 75%). The tree is midpoint rooted. The Italian isolates (collected in Rome) are shown in bold. The figure is also available online as supplementary material in an enlarged version.

Maximum likelihood phylogenetic tree of the third dataset (17-kDa antigen partial gene sequences) was shown in *Figure 4*. *Rickettsia* sp. strain RM1 isolates were found in a statistically supported cluster, which included *Rickettsia* sp. TwKM01 from Taiwan (GenBank accession number AY445821), *R. massiliae* MTU5 (GenBank accession number CP000683), *R. rhipicephali* (GenBank accession number U11020), *R. rhipicephali* (GenBank accession number DQ865207) and *Rickettsia* sp. R300 (GenBank accession number AY472039), both isolated in Brazil isolated, and.

Maximum likelihood analysis identified the *Rickettsia* sp. strain RM2 isolate in a statistically supported cluster with a strain of *Rickettsia* sp. InR/D372 from Korea (GenBank accession number KC888948), two strains from Italy *Rickettsia* sp. IrITA2 and IrITA3 (GenBank accession number AJ427882 and AJ427883), a strain *R. monacensis* IrR/Munich (GenBank accession number EF380355), one strain *Rickettsia* sp. clone Pampulha from Brazil (GenBank accession number JN190456), a *Rickettsia sp.*TR-39 from USA (GenBank accession number DQ480762), two strains of rickettsia endosymbiont of *I. scapularis* from USA (GenBank accession number KC003472 and KC003474), one strain of *Rickettsia tamurae* collected from Japan (GenBank accession number AB114825), a strain *Rickettsia* sp. Ae-8 (GenBank accession number DQ365986), and a strain *Rickettsia* sp. 777c from Australia (GenBank accession number EU283838).

DISCUSSION

Worldwide, ticks are important vectors of human and animal pathogens and a variety of tick-borne infections are considered of medical interest. Several European studies conducted in ticks revealed that the prevalence

of *Rickettsia* SFG ranges from about 3% to 15% [21, 22].

Tick-borne pathogens can occur not only in natural woodlands, but also in recreational urban areas [23-26]. However, to the best of our knowledge, only one investigation was conducted in public parks in Italy, showing the presence of *Bartonella* spp., *B. burgdorferi* s.l., and *Rickettsia* spp. [27].

In view of this fact, we planned a one-year survey to investigate *Rickettsia* spp. Pathogens in ticks collected in the Insugherata Natural Reserve of Rome located in the north-western outskirts of the city. The main tick species found were *R. turanicus* and *I. ricinus* [9], which are well-known vectors of several animal and human pathogens recognized in Italy [28-31].

The results of our study in questing ticks demonstrated an expected occurrence of *Rickettsia* SFG, as previously described [2].

Although the presence of rickettsia in ticks is expected, our results document for the first time the detection of these agents, in an urban park of Italy. In particular, three human pathogens (*R. conorii, R. massiliae* and *R. aeschlimannii*), and two putative new strains with unknown pathogenicity *Rickettsia* sp. strain RM1 obtained from seven individual *R. turanicus*, and *Rickettsia* sp. strain RM2 found in one *I. ricinus* tick, were detected.

The sequence analyses of partial gene sequences of *omp*A, *glt*A and 17-kd antigen of *Rickettsia* sp. strain RM1 showed a high identity with *Rickettsia* sp. strain TwKM01 from Taiwan (99% identity with all partial genes sequenced), while *Rickettsia* sp. strain RM2 exhibited identity with three different strains, *Rickettsia* sp. strain TwKM01 from Taiwan (99% identity with *omp*A partial gene), *Rickettsia* sp. IRS4 collected in Slovakia (100% identity with *gtl*A partial gene) and *Rickettsia* sp. 777c from Australia (99% identity with 17-kd partial gene).

Although the *omp*A phylogenetic analyses showed that, the *Rickettsia* sp. strain RM1 and strain RM2 were most closely related to several rickettsiae with unidentified pathogenicity, the clusters obtained with the *gtl*A and 17-kd sequence analyses were included in statistically supported clades with representative other rickettsiae can cause human diseases. In particular, *Rickettsia* sp. strain RM1 was included in a clade with *R. massiliae,* while *Rickettsia* sp. strain RM2 clustered with *R. monacensis*.

Even if not all rickettsiae detected in this study may be considered human pathogens, their infectivity and potential pathogenicity remains to be further examined. Actually, several *Rickettsia* spp., originally detected in ticks and characterized as unknown pathogenicity, were subsequently demonstrated to be human pathogens, as reported for *R. massiliae* and *R. monacensis* [4, 8].

Moreover, the nonspecific feeding habits of these ticks with the involvement of a wide variety of vertebrates, which are potential reservoirs for several tick-borne pathogens, highlight the potential risk of transmission of multiple infections. In the context of public health, the clinical implications of tick-borne polymicrobial infections may be crucial for planning prophylactic measures and for limiting the probability of misdiagnosis.

CONCLUSION

Our data suggest that atypical cases of rickettsiosis due to agents other than *R. conorii* might occur. Microbiologists and clinicians should be alerted about the presence of new species of rickettsiae in our Country, especially in public parks located in an urban area. For that reason, continuing entomological surveys supported by clinical investigations and identification of rickettsiae in patients, through blood specimens and swabbing eschars analyses, could be an essential aspect to characterize distinct tick-borne rickettsioses occurring in Italy.

However, the epidemiological significance of these results must be taken with prudence, because the presence of a pathogen in ticks does not necessarily mean certain transmission to susceptible hosts. In spite of this, our investigation may be important and helpful for further epidemiological studies of tick-borne pathogens in urban areas in Italy and for the risk prevention associated with tick-borne pathogens transmission to humans and animals.

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Conflict of interest statement

No competing financial interests exist.

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REFERENCES

1. Brouqui P, Parola P, Fournier PE, Raoult D. Spotted fever rickettsioses in southern and Eastern Europe. *FEMS Immunol Med Microbiol* 2007;49:2-12.
2. Ciceroni L, Pinto A, Ciarrocchi S, Ciervo A. Current knowledge of rickettsial diseases in Italy. *Ann N Y Acad Sci* 2006;1078:143-9.
3. Beninati T, Genchi C, Torina A, Caracappa S, Bandi C, Lo N. *Rickettsiae* in ixodid ticks, Sicily. *Emerg Infect Dis* 2005;11:509-11.
4. Madeddu G, Mancini F, Caddeo A, Ciervo A, Babudieri S, Maida I, Fiori ML, Rezza G, Mura MS. *Rickettsia monacensis* as cause of Mediterranean spotted fever-like illness, Italy. *Emerg Infect Dis* 2012;18:702-4. DOI: 10.3201/eid1804.111583.

5. Mura A, Masala G, Tola S, Satta G, Fois F, Piras P, Rolain JM, Raoult D, Parola P. First direct detection of rickettsial pathogens and a new rickettsia "Candidatus *Rickettsia barbariae*" in ticks from Sardinia, Italy. *Clin Microbiol Infect* 2008;14:1028-33. DOI: 10.1111/j.1469-0691.2008.02082.x.
6. Selmi M, Bertolotti L, Tomassone L, Mannelli A. *Rickettsia slovaca* in *Dermacentor marginatus* and tick-borne lymphadenopathy, Tuscany, Italy. *Emerg Infect Dis* 2008;14:817-20. DOI: 10.3201/eid1405.070976.
7. Selmi M, Martello E, Bertolotti L, Bisanzio D, Tomassone L. *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor marginatus* ticks collected on wild boars in Tuscany, Italy. *J Med Entomol* 2009;46:1490-3.
8. Vitale G, Mansueto S, Rolain JM, Raoult D. *Rickettsia massiliae* human isolation. *Emerg Infect Dis* 2006;12:174-5.
9. Di Luca M, Toma L, Bianchi R, Quarchioni E, Marini L, Mancini F, Ciervo A, Khoury C. Seasonal dynamics of tick species in a urban park of Rome. *Ticks Tick-borne Dis* 2013;4:513-7. DOI: 10.1016/j.ttbdis.2013.06.008.
10. Manilla G. *Acari Ixodida* Fauna d'Italia Vol 36. Bologna: Calderini Editore; 1998.
11. Regnery RL, Spruill CL, Plikaytis BD. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J Bacteriol* 1991;173:1576-89.
12. Lee JH, Park HS, Jang WJ, Koh SE, Kim JM, Shim SK, Park MY, Kim YW, Kim BJ, Kook YH, Park KH, Lee SH. Differentiation of rickettsiae by *groEL* gene analysis. *J Clin Microbiol* 2003;41:2952-60.
13. Roux V, Rydkina E, Eremeeva M, Raoult D. Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. *Int J Syst Bacteriol* 1997;47:252-61.
14. Fournier PE, Roux V, Raoult D. Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA. *Int J Syst Bacteriol* 1998;48:839-49.
15. Tsui PY, Tsai KH, Weng MH, Hung YW, Liu YT, Hu KY, Lien JC, Lin PR, Shaio MF, Wang HC, Ji DD. Molecular detection and characterization of spotted fever group rickettsiae in Taiwan. *Am J Trop Med Hyg* 2007;77:883-90.
16. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997;25:4876-82.
17. Hall TA. Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. *Nucleic Acids Symp Ser* 1999;41:95-8.
18. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 1998;14:817-8.
19. Strimmer K, von Haeseler A. Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment. *Proc Natl Acad Sci USA* 1997;94:6815-9.
20. Guindon S, Dufayard J, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst Biol* 2010;59:307-21. DOI: 10.1093/sysbio/syq010.
21. Movila A, Reye AL, Dubinina HV, Tolstenkov OO, Toderas I, Hübschen JM, Muller CP, Alekseev AN. Detection of *Babesia* sp. EU1 and Members of spotted fever group *Rickettsiae* in ticks collected from migratory birds at Curonian Spit, North-Western Russia. *Vector Borne Zoonotic Dis* 2011;11:89-91. DOI: 10.1089/vbz.2010.0043.
22. Stanczak J. The occurrence of Spotted Fever Group (SFG) Rickettsiae in *Ixodes ricinus* ticks (Acari: Ixodidae) in northern Poland. *Ann N Y Acad Sci* 2006;1078:512-4.
23. Reye AL, Hübschen JM, Sausy A, Muller CP. Prevalence and seasonality of tick-borne pathogens in questing *Ixodes ricinus* ticks from Luxembourg. *Appl Environ Microbiol* 2010;76:2923-31. DOI: 10.1128/AEM.03061-09.
24. Schorn S, Pfister K, Reulen H, Mahling M, Silaghi C. Occurrence of *Babesia* spp., *Rickettsia* spp. and *Bartonella* spp. in *Ixodes ricinus* in Bavarian public parks, Germany. *Parasit Vectors* 2011;4:135. DOI: 10.1186/1756-3305-4-135.
25. Stańczak J, Gabre RM, Kruminis-Łozowska W, Racewicz M, Kubica-Biernat B. *Ixodes ricinus* as a vector of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in urban and suburban forests. *Ann Agric Environ Med* 2004;11:109-14.
26. Wielinga PR, Gaasenbeek C, Fonville M, de Boer A, de Vries A, Dimmers W, Akkerhuis Op Jagers G, Schouls LM, Borgsteede F, van der Giessen JW. Longitudinal analysis of tick densities and *Borrelia*, *Anaplasma*, and *Ehrlichia* infections of *Ixodes ricinus* ticks in different habitat areas in The Netherlands. *Appl Environ Microbiol* 2006;72:7594-601.
27. Corrain R, Drigo M, Fenati M, Menandro ML, Mondin A, Pasotto D, Martini M. Study on ticks and tick-borne zoonoses in public parks in Italy. *Zoonoses Public Health* 2012;6:468-76. DOI: 10.1111/j.1863-2378.2012.01490.x.
28. Bertolotti L, Tomassone L, Tramuta C, Grego E, Amore G, Ambrogi C, Nebbia P, Mannelli A. *Borrelia lusitaniae* and spotted fever group rickettsiae *in Ixodes ricinus* (Acari: Ixodidae) in Tuscany, central Italy. *J Med Entomol* 2006;43:159-65.
29. Floris R, Yurtman AN, Margoni EF, Mignozzi K, Boemo B, Altobelli A, Cinco M. Detection and identification of *Rickettsia* species in the northeast of Italy. *Vector Borne Zoonotic Dis* 2008;8:777-82. DOI: 10.1089/vbz.2008.0006.
30. Piccolin G, Benedetti G, Doglioni C, Lorenzato C, Mancuso S, Papa N, Pitton L, Ramon MC, Zasio C, Berciato G. A study of the presence of *B. burgdorferi, Anaplasma* (previously Ehrlichia) *phagocytophilum*, *Rickettsia*, and *Babesia* in *Ixodes ricinus* collected within the territory of Belluno, Italy. *Vector Borne Zoonotic Dis* 2006;6:24-31.
31. Torina A, Alongi A, Scimeca S, Vicente J, Caracappa S, de la Fuente J. Prevalence of tick-borne pathogens in ticks in Sicily. *Transbound Emerg Dis* 2010;57:46-8. DOI: 10.1111/j.1469-0691.2008.02268.x.