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A retrospective study of the characterization of *Rickettsia* species in ticks collected from humans

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ABSTRACT

Rickettsiae (family Rickettsiaceae, order Rickettsiales) are obligate intracellular bacteria transmitted by arthropod vectors. Several *Rickettsia* species causing vector-borne rickettsioses belong to the spotted fever group (SFG). Traditionally, *Rickettsia conorii* has been considered as the main etiologic agent of Mediterranean spotted fever. However, the molecular characterization of rickettsiae allowed identifying other species involved in spotted fever in the Mediterranean region.

In this study, 42 ticks collected from humans were subjected to morphological identification and molecular characterization of *Rickettsia* species potentially involved in human rickettsiosis in Sicily.

Fourteen ticks positive to at least two *Rickettsia* spp. molecular markers were used in the study. Identified *Rickettsia* spp. included *R. conorii*, found in *Rhipicephalus sanguineus* sensu lato and *Rhipicephalus turanicus*, *Rickettsia aeschlimannii* found in *Hyalomma marginatum*, *Hyalomma lusitanicum*, *Dermacentor marginatus* and *Ixodes ricinus*, *Rickettsia massiliae* found in *R. turanicus* and *R. sanguineus s.l.*, and *Rickettsia slovaca* found in *D. marginatus* and *R. sanguineus s.l.*

Our results showed a great variety of zoonotic *Rickettsia* spp. in ticks collected from humans in Sicily. The *Rickettsia* spp. reported in this study were identified in previously recognized or new potential tick vectors in Europe, highlighting the risk of infection by different *Rickettsia* spp. for humans bitten by ticks in Sicily.

1. Introduction

In the last years, several bacterial, viral and parasitic diseases affecting humans and animals have emerged in Europe. Many of the etiological agents of these diseases are transmitted by arthropod vectors (de la Fuente et al., 2008). This phenomenon may be associated with social, ecological, environmental, and microbial risk factors, which act synergistically to facilitate emergence of these pathogens in Europe. Hard ticks (Acari: Ixodidae) are able to transmit pathogens such as viruses, bacteria and protozoa through their bite to humans and animals, and may serve as reservoirs and/or amplifiers for most of these species (Estrada-Peña et al., 2014; Gortazar et al., 2014).

The prokaryotic microorganisms of the order Rickettsiales (genera *Rickettsia*, *Anaplasma*, *Ehrlichia*) are agents of important diseases. These pathogens are transmitted by arthropod vectors and many of them can constitute a risk not only for animals but also for humans (de la Fuente et al., 2008). Rickettsiae (family Rickettsiaceae, order Rickettsiales) are obligate intracellular bacteria transmitted by ticks, fleas, lice and mites. Members of the genus *Rickettsia* may be classified into spotted fever group (SFG) rickettsiae, typhus group rickettsiae, the *Rickettsia bellii* group, and the *Rickettsia canadensis* group (Parola et al., 2013). Several *Rickettsia* spp. causing vector-borne rickettsiosis belong to the spotted

Abbreviations: DEBONEL, *Dermacentor*-borne Necrosis Erythema and Lymphadenopathy; *gltA*, citrate synthase; MSF, Mediterranean spotted fever; *OmpA*, outer membrane protein A; *OmpB*, outer membrane protein B; PCR, polymerase chain reaction; SENLAT, scalp escar neck lymphadenopathy; SFG, spotted fever group; *s.l.*, sensu lato; TIBOLA, tick-borne lymphadenopathy

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fever group (SFG), which represents one of the oldest-known vector-borne zoonosis (Parola et al., 2013).

In the Mediterranean area, *Rickettsia conorii,* comprising a variety of genospecies (Zhu et al., 2005), was traditionally considered as the main etiologic agent of the Mediterranean spotted fever (MSF). MSF is widely distributed through southern Europe, Africa and the Middle East, and it is an emerging or reemerging disease in some regions, while in some other countries of the Mediterranean basin incidence of MSF has increased in the past 10 years (Duque et al., 2012). However, in recent years, the amplification and sequencing of different molecular markers allowed the molecular characterization of strains, and the identification of many new *Rickettsia* spp. or subspecies within the SFG group involved in human rickettsiosis and considered as emerging pathogens (Kernif et al., 2016). They include *R. slovaca*, implicated in development of tick-borne lymphadenopathy (TIBOLA) or *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL) in humans (Cazorla et al., 2003), *R. helvetica* (Fournier et al., 2004), *R. aeschlimannii* (Raoult et al., 2002), *R. massiliae* (Beati and Raoult, 1993; Vitale et al., 2006), and *R. monacensis* (Jado et al., 2007; Simser et al., 2002). Other new *Rickettsia* spp. include *R. sibirica* sensu stricto (Shpynov et al., 2006), *R. heilongjiangensis* (Shpynov et al., 2006), *R. mongolotimonae* (Psaroulaki et al., 2005), and *R. akari* (Radulovic et al., 1996). Recently, *R. felis* was also described as an emerging pathogen of medical importance (Perez-Osorio et al., 2008) also present in Sicily (Giudice et al., 2014).

The objective of this study was the molecular identification and characterization of *Rickettsia* spp. in ticks collected from humans through the use of a multi-gene assay for the amplification and sequencing of different molecular markers. These findings highlighted the importance of the molecular characterization of *Rickettsia* spp. in ticks collected from humans.

2. Material and methods

2.1. Tick collection and identification

During the years 2012 and 2013, 42 individuals have been bitten by ticks in the metropolitan city of Messina, in the Northeastern part of Sicily (Italy). They contacted the Policlinic Hospital of Messina, at the Complex Operative Unit of Infectious Diseases and the ticks were removed and collected from them. The area is characterized by a Mediterranean temperate climate and by a territory mostly mountainous, with some alluvial plains at the mouths of rivers. Only one of the individuals showed clinical manifestations of rickettsiosis. The rest of the individuals were asymptomatic. Collected ticks were stored in alcohol and identified using appropriate taxonomic keys (Apanaskevich et al., 2008; Manilla, 1998; Nava et al., 2015; Walker et al., 2000).

In case the collected ticks were in a state of preservation not suitable for morphological identification at the species level, they were subjected to molecular analysis for species identification. For this purpose, ticks were sectioned longitudinally and one half of each tick was used for DNA extraction. Tick halves were incubated overnight in 180 µl of Genomic Digestion Buffer and 20 µl of proteinase K to digest tick tissues. DNA was extracted using the PureLink Genomic DNA kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The remaining half of each tick was preserved in alcohol. The extracted nucleic acids were analyzed by PCR amplifying a 360 bp fragment of the mitochondrial 12S rDNA (Beati and Keirans, 2001).

2.2. Rickettsia identification and analysis

The extracted nucleic acids were analyzed by polymerase chain reactions (PCR) targeting the *outer membrane protein A* (*OmpA)* (Oteo et al., 2006), *outer membrane protein B* (*OmpB)* (Choi et al., 2005) and the *citrate synthase* (*gltA)* (Roux et al., 1997) genes to detect the presence of DNA from *Rickettsia* spp. (Table 1). Each PCR reaction included a positive control, consisting of DNA from *Rickettsia conorii* Malish 7 strain cultured in VERO cells, and a negative control without DNA. PCRs were carried out in an MJ Research PTC-200 Peltier thermal cycler. PCR products were visualized after electrophoretic migration on a 1.5% agarose gel. PCR products were purified using commercial kits following the manufacturer's procedures, quantified and sent for sequencing to Macrogen Inc. (Macrogen Europe, Amsterdam, The Netherlands).

Obtained sequences were analyzed using Bioedit (Ibis Biosciences, Carlsbad, CA, USA) and ClustalW version 2.0.10 (www.ebi.ac.uk/clustalw) for nucleotide sequence identity to the reference strains reported in the GenBank. The Basic Local Alignment Search Tool (BLAST), DAMBE (http://dambe.bio.uottawa.ca/dambe.asp) and MEGA (www.megasoftware.net) software were used to obtain similarity percentages among the analyzed sequences. Neighbour-joining method was used for phylogenetic analysis using Clustal W. Obtained sequences were submitted to GenBank (accession numbers KT861865-KT861892).

3. Results

3.1. Eight tick species collected from humans in Sicily

Morphological tick identification allowed identifying the following tick species collected from humans in Sicily: *Rhipicephalus turanicus* (N = 13), *Hyalomma lusitanicum* (N = 11), *Rhipicephalus sanguineus* sensu lato (N = 7), *Dermacentor marginatus* (N = 4), *Haemaphysalis punctata* (N = 3), *Hyalomma marginatum* (N = 2), *Ixodes ricinus* (N = 1), and *Rhipicephalus* sp. (N = 1). For the *Rhipicephalus* sp. tick, morphological identification at species level was not possible due to its preservation state. This tick was identified as *R. bursa* by molecular techniques. Obtained sequence was submitted to GenBank (accession number KU512950).

3.2. Thirty three percent prevalence of SFG Rickettsia in ticks collected from humans in Sicily

Of the 42 tick samples, 14 (33%) resulted positive for at least two of

Table 1
PCR performed in this study for the amplification of different *Rickettsia* spp. molecular targets.

Target	Primers	Fragment length	Reference
OmpA	Rr190.70p ATGGCGAATATTTCTCCAAAA Rr190.701n GTTCCGTTAATGGCAGCATCT Rr190.602n AGTGCAGCATTCGCTCCCCCT	631 bp (first) 631 bp (semi-nested)	Oteo et al. (2006)
OmpB	rompB OF GTAACCGGAAGTAATCGTTTCGTAA rompB OR GCTTTATAACCAGCTAAACCACC rompB SFG IF GTTTAATACGTGCTGCTAACCAA rompB SFG IR GGTTTGGCCCATATACCATAAG	511 bp (first) 425 bp (nested)	Choi et al. (2005)
gltA	RpCS.877p GGGGGCCTGCTCACGGCGG RpCS.1258n ATTGCAAAAAGTACAGTGAACA	381 bp	Roux et al. (1997)

Table 2
Rickettsia spp. identified in ticks collected from humans in Sicily and GenBank accession numbers for the *Rickettsia ompA* and *ompB* sequences.

Identified *Rickettsia* spp.	No.	Tick species	Sample ID	*GenBank ompA Accession Number*	*GenBank ompB Accession Number*
Ricketttsia aeschlimannii		*D. marginatus*	*Cerao_Dermacentor_marginatus*	KT861866	KT861880
		H. marginatum	*155_Hyalomma_marginatum*	KT861865	KT861879
	5	*H. marginatum*	*170_Hyalomma_marginatum*	KT861867	KT861881
		H. lusitanicum	*178_Hyalomma_lusitanicum*	KT861868	KT861882
		I. ricinus	*131_Ixodes_ricinus*	KT861869	KT861883
		R. turanicus	*135_Rhipicephalus_turanicus*	KT861870	KT861884
		R. turanicus	*136_Rhipicephalus_turanicus*	KT861873	KT861886
Rickettsia massiliae	*5*	*R. turanicus*	*145_Rhipicephalus_turanicus*	KT861871	KT861887
		R. turanicus	*165_Rhipicephalus_turanicus*	KT861874	KT861888
		R. sanguineus s.l.	*137_Rhipicephalus_sanguineus*	KT861872	KT861885
Rickettsia conorii	*2*	*R. turanicus*	*167_Rhipicephalus_turanicus*	KT861877	KT861891
		R. sanguineus s.l.	*172_Rhipicephalus_sanguineus*	KT861878	KT861892
Rickettsia slovaca	*2*	*R. sanguineus* s.l.	*159_Rhipicephalus_sanguineus*	KT861876	KT861890
		D. marginatus	*157_Dermacentor_marginatus*	KT861875	KT861889

the *Rickettsia* spp. molecular markers used in the study (*ompA*, *ompB* and *gltA*). Positive PCR products were sequenced and the analysis of obtained sequences allowed identifying *R. conorii*, detected in *R. sanguineus* s.l. and *R. turanicus*, and other SFG *Rickettsia* (Table 2). *R. aeschlimannii* was found in five ticks belonging to the species *H. marginatum*, *H. lusitanicum*, *D. marginatus* and *I. ricinus*. *R. massiliae* was detected in four *R. turanicus* ticks and in *R. sanguineus* s.l., while *R. slovaca* was identified in *D. marginatus* and *R. sanguineus* s.l. Phylogenetic multilocus analysis with *ompA–ompB* sequences (GenBank accession numbers KT861865-KT861892; Table 2) confirmed the identity of the *Rickettsia* spp. identified in this study (Fig. 1).

4. Discussion

A great variety of zoonotic *Rickettsia* spp. were identified in this study in the ticks collected from humans in Sicily. The main agent of MSF, *R. conorii,* was detected in only two ticks belonging to the species *R. sanguineus* s.l. and *R. turanicus*. *R. sanguineus* s.l. is extensively recognized as the main vector of *Rickettsia conorii*. However, the role of *R. turanicus* as vector of *R. conorii* has not yet been proven (Parola et al., 2013), even if findings of the pathogen in this tick species have been reported, also in Italy (Mancini et al., 2015). In our study, *R. turanicus* was collected from the only symptomatic patient. To the best of our knowledge, this is the first report of the possible vector role of *R. turanicus* in the transmission of *R. conorii*.

R. aeschlimannii was identified in known (*H. marginatum* and *I. ricinus*; Parola et al., 2013) and potentially new (*D. marginatus* and *H. lusitanicum*) tick vectors of this *Rickettsia* sp. *R. aeschlimannii* was first described in 1997 in *H. marginatum* ticks from Morocco (Beati et al., 1997), and later detected in ticks from Niger, Zimbabwe, and Mali (Parola et al., 2001). In Europe, *R. aeschlimannii* is mainly associated with ticks belonging to the genus *Hyalomma*, and was identified in 2002 in a patient with MSF-like illness (Raoult et al., 2002). Since then, other reports of *R. aeschlimannii* infection were described in patients from South Africa (Pretorius and Birtles, 2002) and Greece (Germanakis et al., 2013). The pathogenicity of this bacterium to humans is not well understood, although MSF-like lesions were reported (Germanakis et al., 2013). Our study emphasizes the risk of rickettsiosis due to *R. aeschlimannii* in Sicily and other Mediterranean countries where *H. marginatum* is present.

R. massiliae is transmitted by tick vectors of the *Rhipicephalus* genus. In Europe, the vectors of this *Rickettsia* species are *R. turanicus*, *R. sanguineus* s.l., *R. bursa*, *R. pusillus* and *I. ricinus* (Parola et al., 2013). Our finding of *R. massiliae* in *Rhipicephalus* ticks collected from humans confirmed this pathogen-vector association and emphasized the possible association between *R. massiliae* and human rickettsiosis in Sicily. In fact, until now, only three cases of human rickettsiosis due to *R. massiliae* have been documented and confirmed by molecular methods in Europe, and two of these cases occurred in Sicily (Cascio et al., 2013; Parola et al., 2008; Vitale et al., 2006). The first case was detected in a blood sample from a patient hospitalized with fever and skin rash in Sicily (Vitale et al., 2006). The second case was a patient who suffered from spotted fever and acute loss of vision in southern France (Parola et al., 2008), and the third case was a child showing scalp eschar and neck lymphadenopathy in Sicily (Cascio et al., 2013).

R. slovaca was originally isolated from *D. marginatus* in Czechoslovakia in 1968, and it is considered the main etiologic agent of TIBOLA (Lakos, 1997), DEBONEL (Oteo and Ibarra, 2002) and SENLAT (scalp escar neck lymphadenopathy) (Angelakis et al., 2010). In Europe, *R. slovaca* is usually associated with *Dermacentor* ticks. *D. marginatus* together with *D. reticulatus* are the recognized vectors for this *Rickettsia* sp. in Europe (Parola et al., 2013). In this study, evidences of *R. slovaca* infection in *D. marginatus* and *R. sanguineus* s.l. ticks collected from humans were reported. While a vector role of *R. sanguineus* s.l. has not yet been proven for *R. slovaca*, the association between *R. slovaca* and *D. marginatus* is widely documented, also in Sicilian ticks (Beninati et al., 2005). Moreover, our findings confirmed previous studies that reported a high risk of rickettsiosis due to *R. slovaca* in Sicily and other Mediterranean countries (Torina et al., 2012).

The results confirmed previous reports showing that several pathogenic *Rickettsia* spp. may be more prevalent than *R. conorii* (Fernández-Soto et al., 2006), although in our study this pathogen was the only causing clinical signs in the individual from whom the tick was collected. All the other individuals did not show clinical manifestations of rickettsiosis. The absence of clinical signs in these individuals could be due to several factors. For example, for *Rickettsia* spp. found in their known vectors such as *R. aeschlimannii* in *I. ricinus* and *H. marginatum*, *R. massiliae* in *R. turanicus* and *R. sanguineus* s.l., *R. conorii* in *R. sanguineus* s.l., and *R. slovaca* in *D. marginatum*, the absence of clinical signs could be due to the fact that ticks were detected and removed soon after attachment and before pathogen transmission. Alternatively, other factors such as pathogen load and host immune response could also affect the clinical outcome. For *Rickettsia* spp. found in new tick spp., vector competence might affect pathogen transmission and disease.

5. Conclusions

The results reported in this study showed that, in addition to *R. conorii,* a variety of *Rickettsia* spp. are present in Sicily. Most of the *Rickettsia* spp. identified in this study were detected in ticks that are considered proven or potential vectors in Europe, suggesting a potential risk of infection by different *Rickettsia* spp. for humans bitten by ticks in

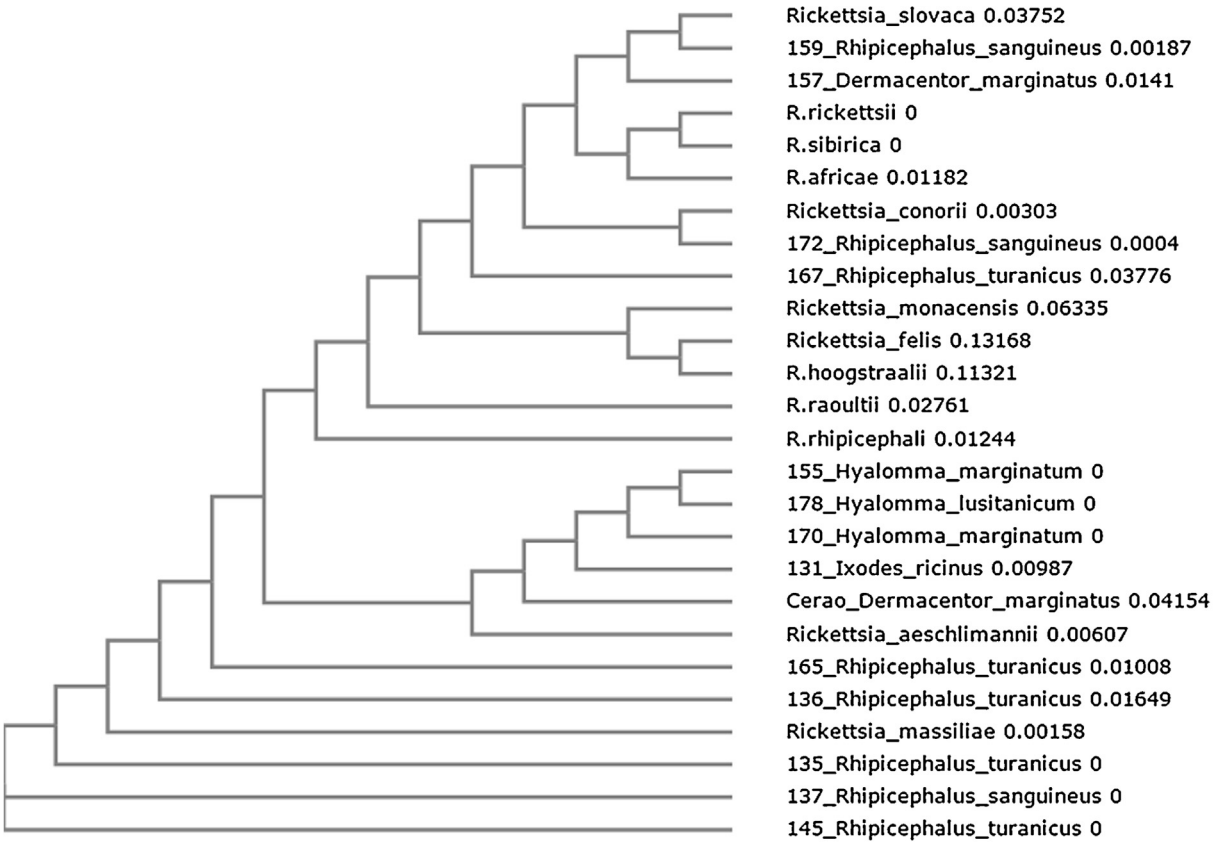

Fig. 1. Phylogenetic analysis of *Rickettsia* spp. The evolutionary history was inferred by using the Neighbor-Joining method for *ompA* and *ompB* genes. Sequences of samples obtained in this study are shown using the identification number of the sample followed by the name of the tick species from which the DNA was isolated (Table 2). For each reference sequence present in GenBank, the name of the *Rickettsia* spp. is shown. Reference sequences included in the analysis are: *Rickettsia aeschlimannii* (*ompA* JF803906.1; *ompB* HQ335156.1), *Rickettsia massiliae* (JQ480842.1; AF123714.1), *Rickettsia slovaca* (EU622810.1; JN182796.1), *Rickettsia conorii* (HM050291.1; JN182801.1), *Rickettsia monacensis* (FJ919651.1; JX683117.1), *Rickettsia felis* (JN990593.1; GQ385243.1).

Sicily. The study reports the first evidence of *R. conorii* possible transmission by *R. turanicus*, suggesting that several still unrecognized tick spp. might be competent vectors for these pathogens. Consequently, it is necessary to use a multidisciplinary approach to characterize the potential tick vectors for SFG rickettsiae. Furthermore, since detected *Rickettsia* spp. may cause clinical rickettsiosis with signs different from those typically associated with MSF caused by *R. conorii*, physicians need to consider the occurrence of atypical signs of rickettsiosis in Sicily.

Declaration of interest

The authors declare that they have no any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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Authors' contributions

AT, SC and AC conceived and supervised the study. FLR, RDA, KR and SS performed the experiments. VB, EG and JF wrote the manuscript. All authors read and approved the final manuscript.

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