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First direct detection of rickettsial pathogens and a new rickettsia, '*Candidatus* Rickettsia barbariae', in ticks from Sardinia, Italy

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ABSTRACT

The present study evaluated the molecular detection and identification of *Rickettsia* species in 83 ticks collected in Sardinia, Italy. Fifteen ticks were PCR-positive using *gltA*-specific and *ompA*-specific primers, leading to the identification of *Rickettsia aeschlimannii* in *Hyalomma marginatum marginatum*, *R. massiliae* in *Rhipicephalus turanicus* and in *Rhipicephalus sanguineus*, and a new rickettsia, previously referred to as PoTiRb169 in Portugal, in four *Rhipicephalus turanicus*. This new species was further characterized by amplification and sequencing of three additional genes (*ompB*, *sca4* and *rrs*). Using the current criteria to name a rickettsia, this uncultivated rickettsia can be given a *Candidatus* status, and we propose to call it '*Candidatus* Rickettsia barbariae'. The detection of three tick-borne rickettsiae in Sardinia raises the possibility that many cases of spotted fever considered by clinicians and health authorities as Mediterranean spotted fever due to *R. conorii* could, in fact, be due to other rickettsiae, including those found in this study. Analysing skin biopsies of inoculation eschars in patients with spotted fever would be, together with continuing entomological surveys, the best way to increase our knowledge of tick-borne rickettsioses in Sardinia and more generally in the Mediterranean basin.

Keywords Italy, rickettsia, Sardinia, spotted fever, ticks

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INTRODUCTION

Tick-borne spotted fever group (SFG) rickettsiae are obligate intracellular, Gram-negative bacteria, commonly associated with ixodid ticks, which can transmit these microorganisms to humans and animals via salivary secretions [1]. Several tick-borne rickettsiae are causative agents of human diseases characterized by various clinical features, including headache, high fever, one or several inoculation eschars (tache noire) at the bite site of the tick and a rash [1].

In Italy, the incidence of tick-borne zoonoses has increased over the last decade. From 1998 to 2002, 4604 clinical cases and 33 deaths were reported to the Health Ministry [2]. The Italian regions with elevated incidences of rickettsial diseases are Sicily, Sardinia, Lazio and Calabria, whereas in other areas, rickettsiosis is sporadic and related to patient stay in an endemic zone. Almost all of the cases reported in Italy are considered as cases of Mediterranean spotted fever (MSF). This disease is due to *Rickettsia conorii* subsp. *conorii* and transmitted by the bite of the brown dog tick, *Rhipicephalus sanguineus* [1]. Until 2002, the only rickettsia isolated from humans and ticks in Italy was *R. conorii*. However, in 2005 the first case of *R. massiliae* was retrospectively documented in a patient from Sicily, Italy [3]. This is the only well-documented case of a human infection with *R. massiliae*. Two additional rickettsial pathogens have been detected in ticks collected in Italy, i.e. *R. aeschlimannii* and *R. slovaca* [4]. Moreover, in 2004, in northern Italy, three cases of a mild form of rickettsiosis were serologically established and

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attributed to *R. helvetica* [5]. The pathogenicity of this rickettsia is still under discussion [1].

Sardinia is the second-largest island in the Mediterranean Sea, with 1.6 million inhabitants, and an area of 24 090 km^2.Its temperate climate (average annual temperature 15–30°C) allows the survival of many kinds of tick vectors. The most common tick species on the island are *Rhipicephalus* spp., *Haemaphysalis* spp. and *Dermacentor* spp. [6].

In Sardinia, the number of notified clinical cases of SFG rickettiosis, presumably MSF, has been 11.9 for every 100 000 inhabitants, in contrast to the national average of 2.1 [7]. To our knowledge, all the studies on rickettsiae conducted in Sardinia have been based on clinical and serological features only. There has been no direct detection or isolation of rickettsiae from ticks or humans in Sardinia. In the present study, a PCR and a nucleotide sequence analysis of amplified products was used to identify rickettsiae in ticks from Sardinia.

MATERIALS AND METHODS

Tick collection

The study was performed in two areas in the north (Sassari) and east (Ogliastra) of Sardinia; both of these regions are characterized by a high prevalence of tick bite-related fever in animal species and a high seroprevalence related to rickettsioses in humans. From June to July 2007, 83 adult ticks were collected from various animals (sheep, goats, horses, cattle and dogs) and from vegetation. All ticks removed from animals were engorged or partially engorged. All ticks were brought to the laboratory of the Institute Zooprofilattico, Sardinia (Sassari) and stored in vials containing 70% ethanol at room temperature until DNA extraction. Samples were thereafter delivered to Marseille, France. The ticks were identified, to the species or genus level, using taxonomic schemes [6].

DNA extraction

Ticks were immersed in distilled water for 10 min, dried on sterile filter paper and crushed individually with a sterile scalpel in Eppendorf tubes. Genomic DNA was extracted using QIAgen columns (QIAamp tissue kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions.

PCR amplification and electrophoresis

PCR was performed using oligonucleotide primers Rp CS. 409p and Rp CS.1258n (Eurogentec, Seraing, Belgium), which amplify a 750-bp fragment of the citrate synthase gene (*gltA*) of *Rickettsia*, as previously reported [8]. The positivity of samples was confirmed by a second PCR using primers Rr 190.70, Rr 190.180 and Rr 190.701 for the *ompA* gene, which amplify a 629–632-bp fragment [9]. In addition, for the detected rickettsiae with homology of <99.9% and 98.8% with the *gltA* and *ompA* genes, respectively, of validated species, three more PCR reactions were performed: (i) a PCR using a battery of primers that amplify a 2400-bp fragment of the rickettsial *ompB* gene; (ii) a PCR using a battery of primers that amplify a 3000-bp fragment of the *sca4* gene; and (iii) a PCR using a battery of primers that amplify a 1500-bp *rrs* gene fragment as previously recommended by the ESCMID Study Group for *Coxiella*, *Anaplasma*, *Rickettsia* and *Bartonella* [10].

A negative control of DNA extracted from non-infected laboratory ticks and a positive control of *R. montanensis* DNA (one negative control for every 20 tested ticks in both cases) were included in each test. Reactions were performed in automated DNA thermal cyclers (GeneAmp PCR System 2400 and 9700; Applied Biosystems, Courtaboeuf, France). PCR products were verified by electrophoresis in agarose (1%) gel stained with ethidium bromide and examined under UV transillumination. PCR products were purified using a QIAquick Spin PCR purification Kit (Qiagen) and sequenced using a DNA sequencing kit (dRhodamine Terminator cycle sequencing ready reaction; Applied Biosystems), according to the manufacturer's instructions. All sequences were assembled and edited with Auto Assembler software (version 1.4; Perkin-Elmer, Courtaboeuf, France) and compared with those of the rickettsiae present in the GenBank database using the BLAST search tool. Sequences obtained were aligned and analysed using the ClustalW and Mega v.4 programs.

RESULTS

The ticks collected were identified as *Rhipicephalus bursa*, *Rhipicephalus turanicus*, *Hyalomma marginatum marginatum* or *Rhipicephalus sanguineus*. One damaged tick was identified to the genus level only, as *Rhipicephalus* sp. (Table 1).

Rickettsial DNA was found in 15 (18%) of the 83 ticks examined using PCR with *gltA*-specific and *ompA*-specific primers (Table 1). Seven of 11 *H. marginatum marginatum* ticks were found to be positive for rickettsial DNA, using both PCR reactions. For all samples, sequence analyses showed 100% similarity with the 750-bp *glt*A fragment and the 630-bp *ompA* fragment of *R. aeschlimannii*. Two of 24 *Rhipicephalus turanicus* ticks contained a rickettsia exhibiting a nucleotide sequence of *gltA* with 99.6% (466/468 bp) similarity to that of *R. massiliae*. The *ompA* sequence showed 99.2% (494/498 bp) similarity to the *ompA* sequence of *R. massiliae*. Both *Rhipicephalus sanguineus* ticks tested positive by PCR. Sequence analysis revealed 100% similarity to the corresponding 750-bp fragment of *gltA* and the 630-bp fragment of *ompA* of *R. massiliae.*

Four of 24 *Rhipicephalus turanicus* ticks were PCR-positive for rickettsial DNA. The *gltA* and *ompA* sequences from all validated species

Table 1. Detection and identification of spotted fever group rickettsiae from ticks, collected in Sardinia, by PCR and sequencing[a]

Tick species (number of tested specimens)	Host	Rickettsial gene targeted/number of ticks positive by PCR/total examined	Animal species with positive ticks	Identification by gene sequence	GenBank accession number
Hyalomma marginatum marginatum (11)	Horses	*gltA* 7/11	Horses	*Rickettsia aeschlimannii* (100%)	U59722.1
		OmpA 7/11		*R. aeschlimannii* (100%)	U43800.1
Rhipicephalus sanguineus (2)	Dogs	*gltA* 2/2	Dogs	*R. massiliae* (100%)	U59720.1
		OmpA 2/2		*R. massiliae* (100%)	U43792.1
Rh. bursa (45)	Goats, cattle, horses, sheep, vegetation	*gltA* 0/45	–	–	–
		OmpA 0/45		–	–
Rh. turanicus (24)	Sheep, cattle, horses, goats	*gltA* 2/24	Cattle, goat	*R. massiliae* (99.6%)	U59719.1
		OmpA 2/24		*R. massiliae* (99.2%)	U43799.1
Rh. turanicus (24)	Sheep, cattle, horses, goats	*gltA* 4/24	Sheep	'*Candidatus* Rickettsia barbariae'[b]	EU272185
		OmpA 4/24		'*Candidatus* Rickettsia barbariae'[c]	EU272186
		OmpB 4/24		'*Candidatus* Rickettsia barbariae'[d]	EU272187
		sca4 4/24		'*Candidatus* Rickettsia barbariae'[e]	EU272188
		rrs 4/24		'*Candidatus* Rickettsia barbariae'[f]	EU272189
Rhipicephalus sp. (1)	Dog	*gltA* 0/1	–	–	–
		OmpA 0/1		–	–

[a]Only ticks positive for *gltA* were tested for *ompA*.
[b]100% similar to the *gltA* sequence of *Rickettsia* PoTiRb169 (DQ423369.1).
[c]100% similar to the *ompA* sequence of *Rickettsia* PoTiRb169 (DQ423366.1).
[d]99.86% similar to the *ompB* sequence of *Rickettsia* PoTiRb169 (DQ423363.1).
[e]98.92% similar to the *sca4* sequence of *Rickettsia africae* (AF151724.2).
[f]99.45% similar to the *rrs* sequences of *Rickettsia conorii* (AE008647.1).

deposited in GenBank were found to be different. They had 100% similarity to the corresponding sequences of an incompletely described rickettsia, temporarily named *Rickettsia* PoTiRb169 (Table 1) [11]. When *ompB* sequences were obtained from the same PCR-positive ticks, they were shown to have 99.86% (774/775 bp) similarity to the *ompB* sequence of *Rickettsia* PoTiRb169. Sequences of the *sca4* and *rrs* genes were obtained and compared with those available in GenBank. Surprisingly, the *sca4* sequence had 98.92% similarity (2841/2872 bp) to that of *R. africae*, whereas the *rrs* sequence had 99.45% (1458/1466 bp) similarity to that of *R. conorii*. No sequences of these two genes of *Rickettsia* PoTiRb169 were available in GenBank. The sequences of the five genes of this rickettsia were obtained from the DNA extracted from the four different *Rhipicephalus turanicus* ticks. The respective sequences obtained from all four ticks were identical.

None of the ticks belonging to the species *Rhipicephalus bursa* were positive for rickettsiae. All the Genbank accession numbers used to compare the sequences obtained are presented in Table 1. The phylogenetic relationships between the rrs, ompB and sca4 genes among the validated Rickettsia species and '*Candidatus* Rickettsia barbariae' are shown in Figure 1.

DISCUSSION

Our results document the first detection in Sardinian ticks of *Rickettsia* spp., including two human pathogens, *R. massiliae* and *R. aeschlimannii*. *R. massiliae* is commonly found in *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* ticks. Since its isolation and characterization in 1993 [12], *R. massiliae* has been found to infect ticks in the European mainland, including France, Greece, Spain [13], Portugal and Switzerland [1]. It has also been detected in engorged female ticks of the *Rhipicephalus sanguineus* group collected in Corsica, a French Mediterranean island [14], as well as in Africa and recently the USA [15]. Trans-stadial and transovarial transmission of *R. massiliae* in *Rhipicephalus sanguineus* group ticks has been reported [14]. Therefore, these ticks can be considered as vectors but also as a reservoir. *Rhipicephalus sanguineus* has become the tick most widely distributed throughout the climatic region of the Mediterranean area, where it feeds on domestic dogs [6]. In the USA, although *R. conorii* has never been described, *Rhipicephalus sanguineus* has been implicated as a vector of *R. rickettsii*, the agent of Rocky Mountain spotted fever [15].

Rhipicephalus turanicus, on the other hand, is distributed in southern Europe and northern

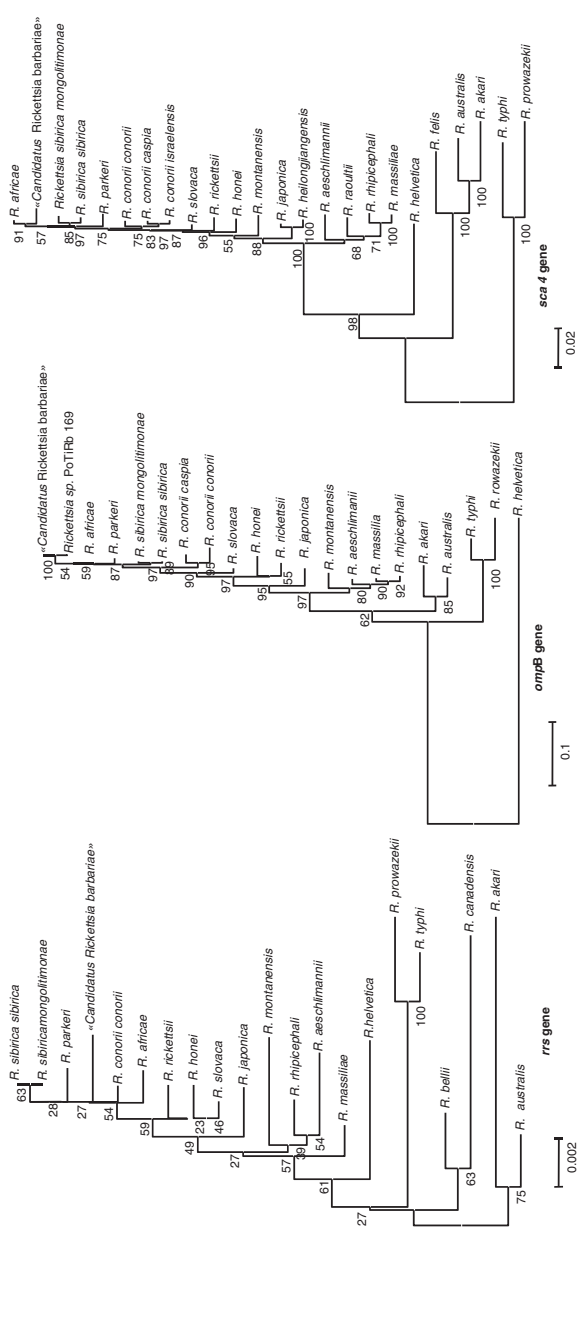

Fig. 1. The trees show the phylogenetic relationships between the *rrs*, *ompB* and *sca4* genes among the validated *Rickettsia* species and '*Candidatus* Rickettsia barbariae'. Sequences were aligned and analysed using the ClustalW and Mega v.4 programmes. Bare scales represent nucleotide sequence divergence. For the phylogenetic tree based on *ompA* genes analyses, see reference [11].

Africa [6]. For 12 years, *R. massiliae* has been considered as a rickettsia of unknown pathogenicity. However, in 2005, *R. massiliae* was reported to be an emerging human pathogen [3].

The second SFG pathogenic rickettsia found in this study was *R. aeschlimannii*. This bacterium has been found in *H. marginatum marginatum* and *H. marginatum rufipes* ticks collected in southern Europe, including Corsica, an island close to Sardinia [16], and Africa, and in *Rhipicephalus appendiculatus* in South Africa [17]. *H. marginatum marginatum* is one of the most important species in the Mediterranean region. It is a vector but also a suspected reservoir of *R. aeschlimannii* [6]. Only two cases of *R. aeschlimannii* infection have been described, including one in a patient bitten in Morocco and one in South Africa [1]. In the first case, the patient presented an inoculation eschar on his ankle, fever, and a generalized maculopapular rash. The second case was that of a patient returning from a trip to South Africa. A *Rhipicephalus appendiculatus* tick was attached to his thigh, and an eschar around the attachment site was noted [17].

We have also detected an incompletely described rickettsia in *Rhipicephalus turanicus* ticks. This rickettsia has recently been detected in Portugal in a *Rhipicephalus bursa* tick, and has provisionally been named PoTiRb169 [11]. The analysis of *gltA*, *ompA* and *ompB* sequences confirmed the presence of this rickettsia in Sardinia. As stated by de Sousa *et al.* [11], when they reported the detection of this bacterium, other gene sequences were required to establish its identity correctly according to the genetic guidelines published by Fournier *et al.* [10,18]. That was done in this study. The sequences of the *rrs* and *sca4* genes were determined and shown to be different from those of all validated *Rickettsia* species (Table 1). Considering these criteria, we propose to give to this strain a *Candidatus* status and name it '*Candidatus* Rickettsia barbariae', with reference to the name *Barbaria* given by Romans to the Mountains of Sardinia.

The data of this study allowed the first identification of rickettsiae in Sardinian ticks, and provide the first evidence of two human pathogens, *R. massiliae* and *R. aeschlimannii*, in this region. The four ticks found to carry rickettsiae had been collected in the same geographical area (Ogliastra). However, DNA detection does not imply transmission competence of the ticks concerned, because they could have been removed from bacteraemic animals. Therefore, further investigations on the relationships between ticks and this new rickettsia are needed.

The results presented here are of epidemiological importance. Indeed, in Sardinia, only *R. conorii conorii*, the agent of MSF, is considered by the medical community, and all reported cases are considered to be MSF. In this study, *R. conorii conorii* was not detected. However, two specimens only of its recognized vector, *Rhipicephalus sanguineus*, have been tested. Furthermore, the rate of infection of *Rhipicephalus sanguineus* by *R. conorii conorii* in nature has been found to be very low in every place where it has been determined [1]. In Sardinia, the incidence of SFG rickettsioses is very high in comparison to that in other regions in Italy. The present data raise the possibility that, in addition to MSF cases, a proportion of the cases reported in Sardinia might be due to other rickettsiae, including *R. massiliae* and *R. aeschlimannii* and also '*Candidatus* Rickettsia barbariae'. The pathogenicity of this rickettsia is as yet unknown. However, as shown recently with other rickettsiae, it could emerge as a human pathogen in the near future.

Clinicians in Sardinia should be informed that several rickettsiae are prevalent on the island. Further studies are needed to better understand rickettsioses in Sardinia by extending entomological surveys with an increased number of tick species and tick specimens. Furthermore, clinical investigations using molecular or culture-based techniques are expected to help identify rickettsiae in patients. PCR and nucleotide sequencing are now used as sensitive and rapid tools for the detection and identification of rickettsiae in blood, and particularly in skin biopsies. Analysing skin biopsies of inoculation eschars in patients with spotted fever would be, together with continuing entomological surveys, the best way to increase our knowledge concerning tick-borne rickettsioses in Sardinia and, more generally, in the Mediterranean basin.

TRANSPARENCY DECLARATION

The authors declare no conflicting or dual interests.

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