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Detection of *Rickettsia* in *Rhipicephalus sanguineus* Ticks and *Ctenocephalides felis* Fleas from Southeastern Tunisia by Reverse Line Blot Assay

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Ticks ($n = 663$) and fleas ($n = 470$) collected from domestic animals from southeastern Tunisia were screened for *Rickettsia* infection using reverse line blot assay. Evidence of spotted fever group *Rickettsia* was obtained. We detected *Rickettsia felis* in fleas, *Rickettsia massiliae* Bar 29 and the *Rickettsia conorii* Israeli spotted fever strain in ticks, and *Rickettsia conorii* subsp. *conorii* and *Rickettsia* spp. in both arthropods. The sensitivity of the adopted technique allowed the identification of a new association between fleas and *R. conorii* subsp. *conorii* species. The presence of these vector-borne *Rickettsia* infections should be considered when diagnosing this disease in humans in Tunisia.

Climate and land-use changes as well as sociodemographic and technological evolution are affecting many biological systems and influencing the emergence and spread of infectious diseases. This has led to an interest in zoonotic vector-borne diseases, including rickettsioses. These emerging infections appear when the obligate intracellular bacteria of the *Rickettsia* genus infect the endothelial cells. They are transmitted to humans and other animals by arthropod bites and are endemic in several regions where they continue to be a health problem with many human cases registered every year. The rickettsial species were widely believed to be restricted to their specific vectors and thus limited to specific areas. However, the detection of many *Rickettsia* species in different vectors in several areas worldwide has changed this assumption (1).

Species in the *Rickettsia* genus are divided into two groups: the spotted fever group (SFG) and the typhus group (TG). These illnesses are caused by about 22 species. Mediterranean spotted fever (MSF) is caused by *Rickettsia conorii* and is a disease described for the first time in Tunisia (2) and the most frequently observed spotted fever infection recorded in public hospitals. However, many clinical, serological, and molecular studies have demonstrated the presence of *R. conorii*, *Rickettsia felis*, and *Rickettsia typhi* in patients (3, 4, 5, 6). A high seroprevalence was also detected among Tunisian blood donors: 8% tested by Western blotting showed the characteristic profile of *R. conorii* (3). Despite the longstanding presence of the rickettsiosis and its medical importance in Tunisia, the arthropod vectors have been poorly investigated.

The detection and isolation of *Rickettsia* sp. require specialized laboratories with a high degree of expertise, primarily because of the species' intracellular life cycle. For this reason, the diagnosis of *Rickettsia* infection is commonly based on clinical human descriptions and serological analyses (5). Several useful and more sensitive molecular tools such as real-time PCR and reverse line blot (RLB) assay have been currently developed to simultaneously detect and identify *Rickettsia* species in hosts and vectors (7).

While serological studies have demonstrated that various *Rickettsia* species circulate in patients in Tunisia, almost no data are available on the potential arthropod vectors. This study was conducted in southeastern Tunisia to identify *Rickettsia* spp. in ar-

thropods (ticks and fleas) likely to be potential vectors of human rickettsial disease, using RLB assay confirmed by sequencing.

MATERIALS AND METHODS

Study. Our study was conducted in the Sfax governorate in southeastern Tunisia, with a population of 1 million inhabitants, or 8.5% of the total Tunisian population. This region, which plays a major role in the Tunisian economy, is situated in the arid bioclimatic zone with the lowest annual rainfall rate (200 mm/year). Agriculture focuses primarily on the cultivation of olive trees. Livestock production is also important, with an estimated 860,000 head of sheep, goats, and cattle in the region's 15 municipalities.

Collection and identification of ticks and fleas. Ticks and fleas, feeding on domestic animals (dogs, sheep, and goats), were manually removed from July to October 2009 in eight municipalities (Sfax Sud, Sfax Ouest, Sakiet Ezzit, Sakiet Eddayer, Agareb, Jebeniana, Malloulech, and Karkenah). All collected specimens were identified to species level using appropriate taxonomic keys (8, 9). The map showing the distribution of arthropods collected from the different municipalities was constructed using ArcGIS 9 (version 9.3.1) software.

DNA extraction and PCR amplification. DNA was extracted from ticks and fleas using the QIAamp tissue DNA kit (Qiagen, Hilden, Germany), preceded by a 3-h treatment with a proteinase K solution (20 mg/ml). Water was included as a negative control to every 20 samples to test for possible contamination. The DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA from each sample between 50 and 400 ng was subjected to PCR assays and RLB hybridization to detect *Rickettsia* spp.

Amplification of rickettsial DNA was performed using primers spanning the 23S-5S intergenic region (forward primer RLB-23S-5-F [5'-GATAGGTCRGRGTGTGGAAGCAC-3'] and reverse primer RLB-23-5-R [biotin-5'-TCGGGAYGGGATCGTGTGTTTC-3']), producing ampli-

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cons of approximately 330 to 533 bp in length (7). PCRs were performed in a thermocycler (PerkinElmer 2400). DNA amplification was done in a 25- μ l reaction volume with 5 mM Tris-HCl-Cl₂Mg, 200 mM each deoxy-nucleoside triphosphate, 0.75 U of *Taq* TaKara DNA polymerase (Applied Biosystems, Branchburg, NJ), with primers used at a final concentration of 5 pmol followed by the addition of 5 μ l of arthropod DNA extract. PCR cycling included an initial denaturing step of 15 min at 94°C, followed by 40 cycles of 1 min at 94°C, 35 s at 60°C, and 1 min at 72°C, with a final elongation of 7 min at 72°C.

Identification of PCR products by RLB hybridization. Briefly, the collected amplicons of the 23S-5S rRNA spacer region of *Rickettsia* were used in a reverse line blot hybridization assay in which specific amino-oligonucleotide probes are covalently linked to activate a membrane in parallel lines using a slotted miniblitter (MN 45). Hybridizations of the denatured PCR product samples with the species-specific probes are detected using chemiluminescence. The membrane was reused a maximum of five times.

The preparation of the RLB membrane and the hybridization of oligonucleotide probes (catch-all *Rickettsia*, SFG, *R. conorii*, *Rickettsia slovaca*, *Rickettsia aeschlimannii*, *Rickettsia rickettsii/sibirica*, *Rickettsia helvetica*, *R. felis*, TG, *R. typhi*, and *Rickettsia prowazekii*) containing a 5' C₁₂ amino linker extremity were carried out as previously described (7).

Positive controls were plasmids containing the 23S-5S intergenic spacer sequence from *R. prowazekii*, *R. conorii*, and *R. aeschlimannii*, kindly provided by Pedro Andra (Centro Nacional de Microbiología, Instituto de Salud Carlos III, Spain), and cell culture DNA of *R. rickettsii*, *R. slovaca*, *R. felis*, and *R. typhi*, kindly provided by Didier Raoult (Unité des rickettsies, Faculté de médecine, Marseille, France). To monitor cross-contamination and false-positive results, negative controls from DNA extraction and PCR amplification were included in each batch tested by the PCR and RLB hybridization.

DNA sequencing and data analysis. To confirm the RLB results, 10 PCR products obtained from 7 ticks and 3 fleas, which were randomly chosen and hybridized with species-specific probes, were sequenced with targeting of the 23S-5S intergenic spacer using RLB-23S-5-F/RLB-23S-5-R primers. In addition, DNA fragments obtained from the 7 ticks were sequenced with targeting of the *ompA* gene using *ompAF/ompAR* primers (10). The DNA of the 3 fleas was sequenced with targeting of the *ompB* gene using M59 and 807R primers (11). Furthermore, samples that hybridized only with the catch-all and SFG probes were sequenced using the same primers.

The sequences from representative rickettsial species chosen to infer the phylogenetic tree of the 23S-5S spacer were aligned using Clustal W Mega 5.02 software. A phylogenetic tree was constructed by the maximum parsimony method using MEGA version 5.02 software.

Statistical analysis. The chi-square test (EpiInfo 6.04) was used to compare rickettsial prevalences in ticks and fleas collected in different municipalities. The observed differences were considered to be significant when the resulting *P* value was less than 0.05.

Nucleotide sequence accession numbers. Sequence data have been deposited in GenBank; accession numbers for the partial 23S-5S intergenic spacer sequences and *ompA* gene are [KF245433](#) to [KF245444](#) and [KF245445](#) to [KF245453](#), respectively.

RESULTS

Collection and identification of ticks and fleas. Eighty-six domestic dogs, sheep, and goats from 8 municipalities of the Sfax governorate were examined for the presence of hematophagous potential arthropod vectors of *Rickettsia*. A total of 663 ticks and 470 fleas were removed from 38 infested animals (16 dogs, 12 sheep, and 10 goats) (Fig. 1). All 663 ticks were identified as *Rhipicephalus sanguineus* (159 males, 400 females, and 104 nymphs), and all fleas were identified as *Ctenocephalides felis* (186 males and 284 females). *R. sanguineus* (*n* = 657) ticks were collected from 13 of the 28 dogs and from a single ovine (*n* = 6). *C. felis* fleas were

removed from 3 dogs (*n* = 40), 11 of the 14 sheep (*n* = 214), and 10 of the 40 goats (*n* = 216).

Detection of rickettsiae in collected arthropods by RLB. Four hundred three of the 1,133 collected arthropods (198 *R. sanguineus* ticks and 205 *C. felis* fleas) were analyzed for the presence of *Rickettsia* DNA.

By RLB, the overall *Rickettsia* prevalence in the ticks and fleas that were analyzed was 22.8% (92/403). A significant difference was observed between the infection rate in *R. sanguineus* ticks, 37.4% (74/198), and that in *C. felis* fleas, 8.3% (17/205) ($\chi^2 = 36.2$, *df* = 1, *P* < 0.001) (Table 1).

R. sanguineus adults had a significantly higher rate of *Rickettsia* infection (69/163, 42.3%) than did the nymphs (5/35, 14.3%) ($\chi^2 = 5$, *P* = 0.001) (Table 1). The *Rickettsia* prevalence in *R. sanguineus* ticks varied among municipalities from 11.1% to 65.2%: the observed difference was statistically significant ($\chi^2 = 27.82$, *P* < 0.001). A total of 74 DNA samples, extracted from *R. sanguineus* ticks, reacted with the *Rickettsia* catch-all and SFG probes (Fig. 2), of which 69 (93.2%) hybridized with *R. conorii* probes while the remaining 5 *Rickettsia* isolates, which did not react with any specific probes, were sequenced.

R. conorii was detected in ticks collected on dogs and in all sites where ticks were sampled (Table 1).

The 17 positive *C. felis* fleas were detected in five of eight sites visited (Table 1). The samples with positive DNA showed hybridization with the catch-all and the specific SFG probes. RLB allowed species identification of 15 flea samples: 13 hybridized with the *R. conorii* probe and 2 with the *R. felis* probe. The two remaining samples, which did not react with any of the specific probes, were sequenced.

R. conorii was detected in *C. felis* fleas collected from sheep and goats from 5 municipalities (Sakiet Ezzit, Sfax Sud, Sfax Ouest, Karkennah, and Malloulech) (Table 1). *R. felis* was identified in the fleas removed from a sheep and a goat in two sites (Sakiet Ezzit and Karkennah).

DNA sequencing and data analysis. Positive samples (5 ticks and 2 fleas) showing no reaction with any of the specific probes were analyzed by sequencing three fragments: the 23S-5S intergenic spacer, the *ompA* gene, and the *ompB* gene. Sequencing allowed the identification of *Rickettsia massiliae* Bar 29 with 100% similarity to the partial 23S-5S sequence of the Spanish strain (AY125014) in two ticks and no fleas. *ompA* gene sequencing for these two infected *R. sanguineus* ticks confirmed the result and also showed 100% similarity with *R. massiliae* Bar 29 (U43792). These two ticks were collected from a sheep in Sakiet Ezzit and a dog in Jebeniana.

The sequencing of the 23S-5S spacer of randomly chosen *R. sanguineus* ticks infected by *R. conorii* confirmed the infection of 5 of 7 ticks by the *R. conorii* Israeli spotted fever strain, which is similar to the published shotgun sequence of *R. conorii* ISF strain ISTT CDC1 contig09 (AJVP01000009.1). These 5 ticks were collected from Jebeniana. These samples yielded positive PCR products for the *ompA* gene with 99% similarity with the Italian strain Israeli tick typhus *Rickettsia* strain 3 (AY197564). The two remaining ticks, collected from dogs in Karkennah and Malloulech, were infected with *R. conorii*, and their sequences targeting the 23S-5S spacer and the *ompA* gene showed 99% similarity with *Rickettsia conorii* subsp. *conorii* (AE006914).

The sequenced PCR product of the 23S-5S spacer obtained from three fleas confirmed the infection of two *C. felis* fleas

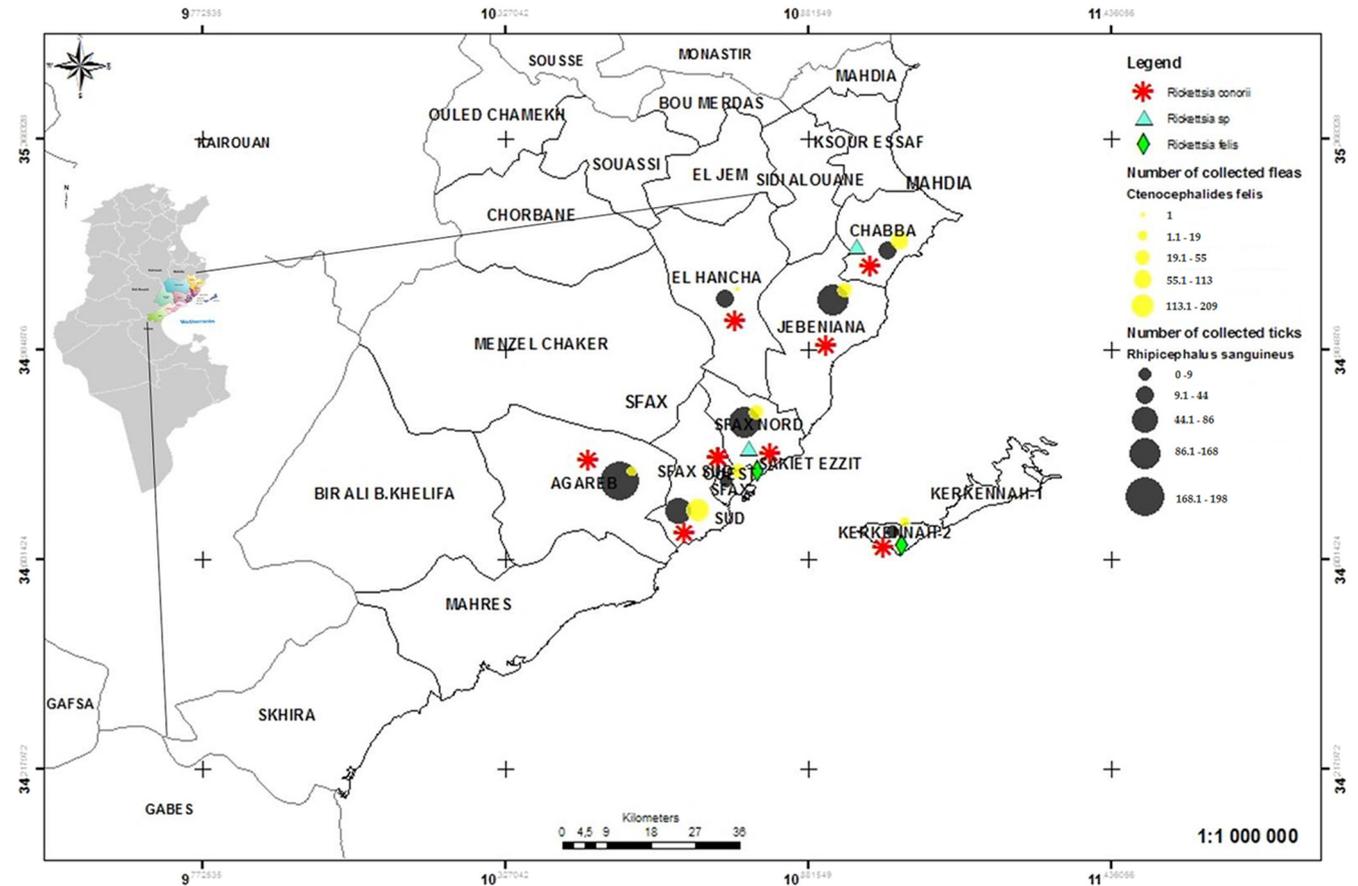


FIG 1 Occurrence of arthropods and *Rickettsia* spp. in Sfax: Sfax region map showing the eight sampling areas and distribution of arthropods and *Rickettsia* species.

by *R. felis* with 100% similarity to the strain URRWFXCa2 (CP000053). These two fleas were collected from sheep in Sakiet Ezzit and Karkennah. The third sequence of a flea, collected from a goat from Sfax Ouest, was 100% identical to the partial sequence of the 23S-5S spacer of *R. conorii* subsp. *conorii* strain Malish 7

(AE006914). The *ompB* sequencing confirmed the species results obtained from 23S-5S sequencing for fleas infected by *R. felis*.

In a maximum parsimony analysis based on the alignment of the 23S-5S spacers of rickettsiae, a phylogenetic tree was constructed from sequences of the *Rickettsia* species and strains ob-

TABLE 1 RLB identification and prevalence of *Rickettsia* in ticks and fleas from Sfax municipalities^a

| Municipality | <i>R. sanguineus</i> | | | | <i>Rickettsia</i> species identified (no.) | <i>C. felis</i> | | | <i>Rickettsia</i> species identified (no.) |
|--------------|----------------------|---------------|-------------|---------------|--|-----------------|------------|---------------|--|
| | Female (n/N) | Male (n/N) | Nymph (n/N) | Total n/N (%) | | Female (n/N) | Male (n/N) | Total n/N (%) | |
| Sakiet Ezzit | 4/23 | 11/29 | — | 15/52 (28.8) | <i>R. conorii</i> (12), SFG (3) | 4/11 | 0/3 | 4/14 (28.6) | <i>R. conorii</i> (3), <i>R. felis</i> (1) |
| Jebeniana | 2/4 | 13/16 | 1/3 | 16/23 (65.2) | <i>R. conorii</i> (15), SFG (1) | 0/12 | 0/4 | 0/16 (0) | 0 |
| Safx Sud | 5/18 | 6/9 | 1/14 | 12/41 (29.3) | <i>R. conorii</i> (12) | 2/32 | 4/52 | 6/84 (17.1) | <i>R. conorii</i> (6) |
| Hincha | 3/5 | 10/17 | 1/1 | 14/23 (60.9) | <i>R. conorii</i> (14) | — | — | — | — |
| Karkennah | — | 5/6 | — | 5/6 (83.3) | <i>R. conorii</i> (5) | 2/8 | — | 2/8 (25) | <i>R. conorii</i> (1), <i>R. felis</i> (1) |
| Malloulech | 0/1 | 0/1 | 2/16 | 2/18 (11.1) | <i>R. conorii</i> (1), SFG (1) | 3/45 | 1/18 | 4/63 (6.3) | SFG (2), <i>R. conorii</i> (2) |
| Sfax Ouest | — | — | — | — | — | 1/4 | 0/4 | 1/8 (12.5) | <i>R. conorii</i> (1) |
| Agareb | 0/1 | 10/33 | 0/1 | 10/35 (28.6) | <i>R. conorii</i> (10) | 0/10 | 0/2 | 0/12 (0) | — |
| Total | 14/52 (26.9) | 55/111 (49.5) | 5/35 (14.3) | 74/198 (37.4) | | 12/122 (9.8) | 9/83 (6) | 17/205 (8.3) | |

^a Symbols and abbreviations: —, no samples; n, number of positive DNA samples; N, number of tested specimens; (%), % prevalence.

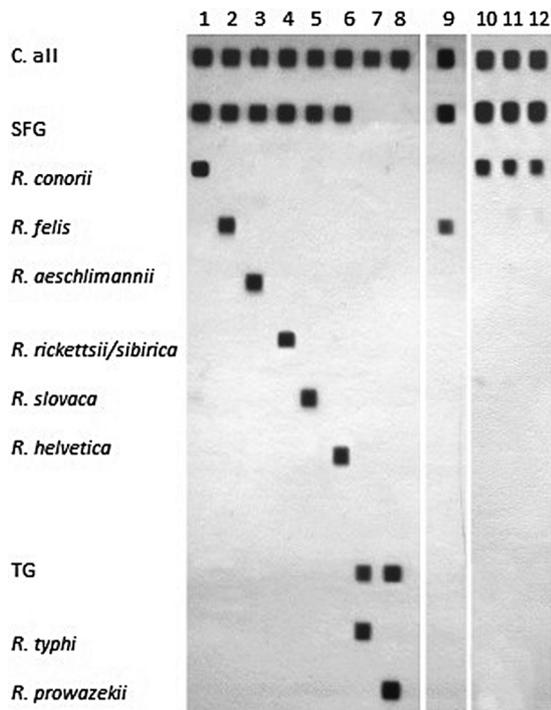


FIG 2 Reverse line blot hybridization assay for the detection and identification of *Rickettsia* spp. in ticks and fleas: membrane carrying species-specific probes. The oligonucleotide probes are attached to the membrane horizontally, and the PCR samples were applied vertically, on the perpendicular. The numbered lanes represent PCR products obtained from the positive control (lanes 1, 2, 3, 4, 5, 6, 7, and 8, *R. conorii*, *R. felis*, *R. aeschlimannii*, *R. rickettsii*, *R. slovaca*, *R. helvetica*, *R. typhi*, and *R. prowazekii*, respectively) and sample-derived PCR products (lanes 9, 10, 11, and 12).

tained from GenBank. Phylogenetic studies currently show that several groups can be determined within the genus *Rickettsia*. Figure 3 shows that sequences from Tunisian samples were clustered in three groups. Six sequences isolated from ticks (KF245435 to KF245440 and KF245442) and one flea (KF245443) were clustered in the first group, which contains most of the SFG *Rickettsia* species (*R. conorii* subsp. *conorii*, *Rickettsia conorii* ISF strain, *Rickettsia sibirica* strain Mongolotimonae, *Rickettsia africae*, *Rickettsia parkeri*, *R. slovaca*, *R. rickettsii*, *Rickettsia honei*, and *Rickettsia japonica*). Two other sequences (KF245434 and KF245444), isolated from two ticks infected by *R. massiliae* Bar 29, were included in the second group (*R. massiliae* Bar 29, *Rickettsia rhipicephali*, *R. aeschlimannii*, and *Rickettsia montanensis*). Finally, sequences from two fleas infected by *R. felis* (KF245433 and KF245441) were clustered in the third group, which contains *R. felis*, *Rickettsia australis*, and *Rickettsia akari*.

DISCUSSION

In this study, using molecular tools (RLB and sequencing), we report evidence of spotted fever group rickettsiae among ectoparasites collected from domestic animals in the Sfax governorate.

Tick and flea species removed from dogs and small ruminants were identified as *Rhipicephalus sanguineus* and *Ctenocephalides felis*. The occurrence of these two arthropods in this region could be attributed to the presence of favorable climatic conditions and adaptation to hosts (dogs, sheep, and goats) (12, 13). *R. sanguineus*, a three-host tick, is mainly collected from dogs, its pre-

ferred host (13), and only a few specimens were found on small ruminants. This tick is well adapted to human rural and urban environments because of its association with dogs, considered to be the main reservoir of *R. conorii* (14). In contrast, *C. felis*, a ubiquitous ectoparasite, was found on all the prospected animals (dogs, sheep, and goats).

To detect *Rickettsia*, several PCRs targeting a variety of genes were described. However, the intergenic spacer 23S-5S rRNA showed higher sensitivity since it has a high copy number in the genome, making it easy to amplify from even small quantities of DNA. Moreover, it is widely used in taxonomy and suitable for the clarification of phylogenetic relationships within the SFG even between closely related species (15).

RLB or PCR-reverse blot hybridization assay was described as a very sensitive and specific test (7) and a fast, reliable technique (16) for the simultaneous detection and identification of pathogens. Using this method, we confirmed that *Rickettsia* infection is prevalent in *R. sanguineus* ticks and *C. felis* fleas collected in the Sfax area. *R. sanguineus* had a significantly higher rate of *Rickettsia* infection than did fleas. This species of tick is considered to be the main vector and reservoir of the *R. conorii* complex in the Mediterranean region: Spain, Italy, Greece, France, and Portugal (17, 18).

The prevalence of *Rickettsia* infection in *R. sanguineus* (37.4%) is roughly similar to that reported in central Spain (25%) (19) but higher than that recorded in North African countries such as Morocco (4.7%) (20) and in some European countries, such as Greece (2.4%) and Cyprus (8%) (21, 22). This large variation among the reported prevalence of *Rickettsia* could be related to the differences in methodologies used, the biotope of the vectors, and the abundance of *Rickettsia* reservoirs. Differences in the prevalence of *Rickettsia* in ticks were observed among the municipalities, with the highest prevalence detected in Karkennah (83.3%), followed by Jebeniana (65.2%). For Karkennah, the results should be confirmed by other sampling since all ticks were collected from the same dog. For Jebeniana, the observed rate may be related to geographic and socio-occupational factors, especially the fact that family incomes were based on livestock. The infection rate of adult ticks was statistically higher than that of nymphs, a fact that was also demonstrated by a xenodiagnostic study (14).

Since the first description of *R. conorii* (in *R. sanguineus* ticks) by Brumpt in 1932 in Tunisia (23), no recorded report has confirmed the presence of this bacterium in ticks. However, *R. conorii* was detected by molecular tools in patients in Tunisia (6, 24). In our study, species-specific probes showed that 34% of DNA samples from *R. sanguineus* ticks, collected from dogs in the Sfax governorate, contained DNA corresponding to *R. conorii*. In Algeria, a neighboring country, Bitam et al. reported an infection rate of 26% for this bacterium in *R. sanguineus* ticks (25), which is higher than those reported in northern Mediterranean regions such as Bulgaria, Turkey, and Albania (1.4%) (26).

In the Mediterranean region, dogs show a high prevalence of infestation with *R. sanguineus* ticks and the close proximity of animals and humans is a risk factor for the *Rickettsia* infection (1). Znazen et al. (27) have reported 37 cases of SFG rickettsiae in the Sfax region based on clinical and serological data. In 19 of these cases, there was reported contact with animals (27). In Morocco, among 45 patients, 34 reported contact with dogs and 29 had *R. conorii* subsp. *conorii* antibodies or a positive skin biopsy specimen (28). In Algeria, Mouffok et al. (29) reported that 161 rick-

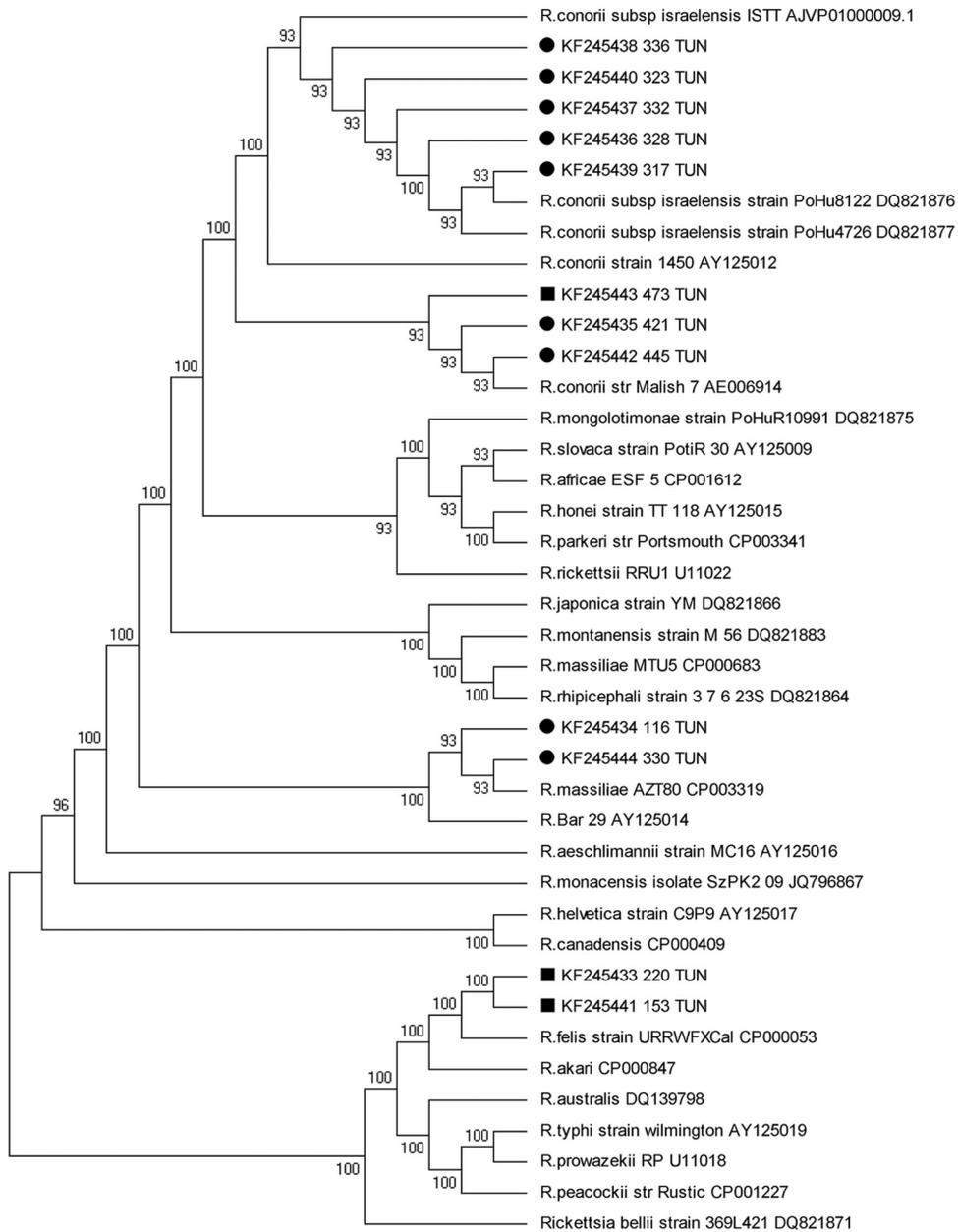


FIG 3 Phylogenetic tree based on the studies of 23S-5S intergenic spacer of bacteria of the genus *Rickettsia* using the MEGA 5.02 software. The tree was obtained using the maximum parsimony method. The numbers at the nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The TUN sequences detected in this work have been deposited in GenBank under accession numbers KF245433 and KF245444, respectively. The sequences used for comparison were obtained from GenBank. TUN, Tunisia; ●, tick samples; ■, flea samples.

ettsiosis cases had been exposed to dogs. Of these, 58% of the patients had an *R. conorii* infection (29). Moreover, in our study, 2 of 6 *R. sanguineus* ticks collected on sheep in Sakiet Ezzit were infected by *R. conorii*. In Cyprus, Chochoiakis et al. (22) also found that the prevalence of infection in *R. sanguineus* ticks collected from sheep and goats was significantly higher than in those from dogs. *R. conorii* has also been detected in several other countries in other tick species such as *Rhipicephalus bursa*, *Dermacentor marginatus*, and *Ixodes ricinus* (17).

In addition to *R. conorii* subsp. *conorii*, *R. sanguineus* ticks were also infected by the *Rickettsia* Israeli spotted fever strain

(AY197564). This *Rickettsia* strain was first isolated from ticks in Palestine in 1946 (30). This strain has been classified with *R. conorii* subsp. *conorii*, *Rickettsia conorii* subsp. *indica*, and *Rickettsia conorii* subsp. *caspi* as a new subspecies within *R. conorii* on the basis of multilocus sequence typing (31). Recently, in Israel, Harus et al. found that 48% of *R. sanguineus* ticks were infected with *Rickettsia* spp., of which 6% were the *R. conorii* ISF strain (32). Moreover, the *Rickettsia conorii* ISF strain has been detected outside Israel in Italy and Portugal (33). Our finding of the strain *R. conorii* ISF in *R. sanguineus* also suggested that the geographical distribution of this species might be wider than previously

thought since it occurs in Tunisia. Indeed, in a recent study, Znazen et al. reported 2 cases of ISF from patients with fever, headache, and arthromyalgia confirmed by the detection of rickettsial DNA in skin biopsy samples (24).

In this study, a third *Rickettsia* species, *R. massiliae* strain Bar 29, was detected in three *R. sanguineus* ticks, collected from sheep and dogs from Sakiet Ezzit and Jebeniana. This *Rickettsia* strain was first isolated in 1996 from *R. sanguineus* in Barcelona, Spain (18). Moreover, a strain close to *R. massiliae* Bar 29 was isolated from *R. sanguineus* ticks collected in Arizona (United States) (34). This strain, which is close to *R. massiliae*, is identical to an isolate previously described as MTU5 and slightly different from *R. massiliae* by antigenic and phenotypic criteria (35). Indeed, this is the first report of *R. massiliae* Bar 29 isolated from *R. sanguineus* in North Africa. However, *R. massiliae* was detected in *R. sanguineus* in Morocco with a prevalence of 4.7% (20) and in Algeria (25). This species, which appears to occur in many countries, was detected in *R. sanguineus* and *Rhipicephalus turanicus* collected in Italy, France, Greece, Spain, and Israel (32).

With respect to *C. felis*, 17 fleas (8.3%) contained *Rickettsia* DNA, including 13 that were hybridized with *R. conorii*. Therefore, we confirmed that in the Sfax region, ticks could transmit MSF disease, as could *C. felis* fleas, which may play an important role. To our knowledge, this is the first report that shows the circulation of the etiological agent of MSF in *C. felis* fleas in the world.

In addition to *R. conorii*, we also detected *R. felis* in two specimens of *C. felis*. *R. felis* has been associated with fleas worldwide, in Algeria and Morocco (37), Italy (38), and the United States (39). However, in this investigation, the prevalence of *R. felis* (1%) is lower than that reported in Morocco (20% in *C. felis* fleas removed from sheep, cats, and dogs [37]) and in Italy (11.9% in dog and cat fleas [38]).

The presence of *R. felis* in fleas collected from domestic animals in the Sfax region is not surprising given that *R. felis* antibodies were found in 8 patients from this region by immunofluorescent assay (IFA) and Western blotting (5). Our results confirm for the first time the presence in Tunisia of *R. felis* in *C. felis* fleas collected from Sakiet Ezzit and Karkennah from sheep and goats, respectively. Indeed, *R. felis* is an emergent rickettsial pathogen for humans with a worldwide distribution (40).

This study provides current data about the rickettsial species and their arthropod vectors that can represent a risk for humans and animals in Tunisia. This is the first report describing the presence of the *R. conorii* ISF strain and *R. massiliae* strain Bar 29 in *R. sanguineus* ticks and *R. conorii* and *R. felis* in *C. felis*, collected in Tunisia. With respect to public health, *R. sanguineus* and *C. felis*, both of which are abundantly present, seem to represent the most significant tick and flea species carrying *Rickettsia* agents and may play an important role in maintaining rickettsial infections. Further investigations in humans and animals are needed and must be compared to these data.

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REFERENCES

1. Raoult D, Roux V. 1997. Rickettsioses as paradigms of new or emerging infectious diseases. *Clin. Microbiol. Rev.* 10:694–719.
2. Conor A, Bruch A. 1910. Une fièvre éruptive observée en Tunisie. *Bull. Soc. Pathol. Exot. Filiales* 8:492–496.
3. Letaïef AO, Yacoub S, Dupont HT, Le Cam C, Ghachem L, Jemni L, Raoult D. 1995. Seroepidemiological survey of rickettsial infections among blood donors in central Tunisia. *Trans. R. Soc. Trop. Med. Hyg.* 89:266–268. [http://dx.doi.org/10.1016/0035-9203\(95\)90531-6](http://dx.doi.org/10.1016/0035-9203(95)90531-6).
4. Letaïef A. 2006. Epidemiology of rickettsioses in North Africa. *Ann. N. Y. Acad. Sci.* 1078:34–41. <http://dx.doi.org/10.1196/annals.1374.004>.
5. Znazen A, Rolain JM, Hamami N, Hamami A, Ben Jemaa M, Raoult D. 2006. Rickettsia felis infections, Tunisia. *Emerg. Infect. Dis.* 12:138–140. <http://dx.doi.org/10.3201/eid1201.050876>.
6. Sfar N, Kaabia N, Letaïef A, Rolain JM, Parola P, Bouattour A, Raoult D. 2009. First molecular detection of *R. conorii* subsp. *conorii* 99 years after the Conor description of Mediterranean spotted fever, in Tunisia. *Clin. Microbiol. Infect.* 15:309–310. <http://dx.doi.org/10.1111/j.1469-0691.2008.02253.x>.
7. Jado I, Escudero R, Gil H, Jiménez-Alonso MI, Sousa R, Garcia-Pérez AL, Rodríguez-Vargas M, Lobo B, Anda P. 2006. Molecular method for identification of Rickettsia species in clinical and environmental samples. *J. Clin. Microbiol.* 44:4572–4576. <http://dx.doi.org/10.1128/JCM.01227-06>.
8. Bouattour A. 2002. Clé dichotomique et identification des tiques (Acari: Ixodidae) parasites du bétail au Maghreb. *Arch. Inst. Pasteur Tunis* 79:43–50.
9. Beaucournu JC, Menier K. 1998. The genus Ctenocephalides Stiles and Collins, 1930 (Siphonaptera, Pulicidae). *Parasite* 5:3–16.
10. Fournier PE, Roux V, Raoult D. 1998. Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA. *Int. J. Syst. Bacteriol.* 48:839–849. <http://dx.doi.org/10.1099/00207713-48-3-839>.
11. Roux V, Raoult D. 2000. Phylogenetic analysis of members of the genus Rickettsia using the gene encoding the outer-membrane protein rOmpB (ompB). *Int. J. Syst. Evol. Microbiol.* 50:1449–1455. <http://dx.doi.org/10.1099/00207713-50-4-1449>.
12. Franc M, Cadiergues MC. 1997. Mode of contamination of dogs by adult fleas (Ctenocephalides felis) in different controlled environments. *Rev. Med. Vet.* 148:23–26.
13. M'ghirbi Y, Bouattour A. 2008. Detection and molecular characterization of Babesia canis vogeli from naturally infected dogs and Rhipicephalus sanguineus ticks in Tunisia. *Vet. Parasitol.* 152:1–7. <http://dx.doi.org/10.1016/j.vetpar.2007.12.018>.
14. Levin ML, Killmaster LF, Zemtsova GE. 2012. Domestic dogs (Canis familiaris) as reservoir hosts for Rickettsia conorii. *Vector Borne Zoonotic Dis.* 12:28–33. <http://dx.doi.org/10.1089/vbz.2011.0684>.
15. Vitorino L, Chelo IM, Bacellar F, Ze-Ze L. 2007. Rickettsiae phylogeny: a multigenic approach. *Microbiology* 153:160–168. <http://dx.doi.org/10.1099/mic.0.2006/001149-0>.
16. Kong F, Gilbert GL. 2006. Multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB)—a practical epidemiological and diagnostic tool. *Nat. Protoc.* 1:2668–2680. <http://dx.doi.org/10.1038/nprot.2006.404>.
17. Parola P, Raoult D. 2001. Ticks and tick-borne bacterial diseases in humans: an emerging infectious threat. *Clin. Infect. Dis.* 32:897–928. (Erratum, 33:749.) <http://dx.doi.org/10.1086/319347>.
18. Parola P, Paddock CD, Raoult D. 2005. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clin. Microbiol. Rev.* 18:719–756. <http://dx.doi.org/10.1128/CMR.18.4.719-756.2005>.
19. Toledo A, Olmeda AS, Escudero R, Jado I, Valcárcel F, Casado-Nistal MA, Rodríguez-Vargas M, Gil H, Anda P. 2009. Tick-borne zoonotic bacteria in ticks collected from central Spain. *Am. J. Trop. Med. Hyg.* 81:67–74.
20. Sarih M, Socolovschi C, Boudebouch N, Hassar M, Raoult D, Parola P. 2008. Spotted fever group rickettsiae in ticks, Morocco. *Emerg. Infect. Dis.* 14:1067–1073. <http://dx.doi.org/10.3201/eid1407.070096>.
21. Psaroulaki A, Spyridaki I, Ioannidis A, Babalis T, Gikas A, Tselentis Y. 2003. First isolation and identification of Rickettsia conorii from ticks

- collected in the region of Fokida in central Greece. *J. Clin. Microbiol.* 41:3317–3319. <http://dx.doi.org/10.1128/JCM.41.7.3317-3319.2003>.
22. Chochlakis D, Ioannou I, Sandalakis V, Dimitriou T, Kassinis N, Papadopoulos B, Tselentis Y, Psaroulaki A. 2012. Spotted fever group rickettsiae in ticks in Cyprus. *Microb. Ecol.* 63:314–323. <http://dx.doi.org/10.1007/s00248-011-9926-4>.
 23. Brumpt E. 1932. Longévitité du virus de la fièvre boutonneuse (*Rickettsia conorii*, n. sp.) chez la tique *Rhipicephalus sanguineus*. *C. R. Soc. Biol.* 110:1119–1202.
 24. Znazen A, Hammami B, Lahiani D, Ben Jemaa M, Hammami A. 2011. Israeli spotted fever, Tunisia. *Emerg. Infect. Dis.* 17:1328–1330. <http://dx.doi.org/10.3201/eid1707.101648>.
 25. Bitam I, Parola P, Matsumoto K, Rolain JM, Baziz B, Boubidi SC, Harrat Z, Belkaid M, Raoult D. 2006. First molecular detection of *R. conorii*, *R. aeschlimannii*, and *R. massiliae* in ticks from Algeria. *Ann. N. Y. Acad. Sci.* 1078:368–372. <http://dx.doi.org/10.1196/annals.1374.073>.
 26. Christova I, van de Pol J, Yazar S, Velo E, Schouls L. 2003. Identification of *Borrelia burgdorferi* sensu lato, *Anaplasma* and *Ehrlichia* species, and spotted fever group rickettsiae in ticks from southeastern Europe. *Eur. J. Clin. Microbiol. Infect. Dis.* 22:535–542. <http://dx.doi.org/10.1007/s10096-003-0988-1>.
 27. Znazen A, Benarab N, Marrekchi C, Benjemma M, Hammami A. 2008. Etude de 50 cas de rickettsioses confirmées par la sérologie au sud de la Tunisie. *Med. Mal. Infect.* 38:S179–S181. [http://dx.doi.org/10.1016/S0399-077X\(08\)73218-9](http://dx.doi.org/10.1016/S0399-077X(08)73218-9).
 28. Meskini M, Beati L, Benslimane A, Raoult D. 1995. Seroepidemiology of rickettsial infections in Morocco. *Eur. J. Epidemiol.* 11:655–660. <http://dx.doi.org/10.1007/BF01720299>.
 29. Mouffok N, Parola P, Lepidi H, Raoult D. 2009. Mediterranean spotted fever in Algeria—new trends. *Inter J. Infect. Dis.* 13:227–235. <http://dx.doi.org/10.1016/j.ijid.2008.06.035>.
 30. Valero A. 1949. Rocky Mountain spotted fever in Palestine. *Harefuah* 36:99.
 31. Zhu Y, Fournier PE, Ereemeeva M, Raoult D. 2005. Proposal to create subspecies of *Rickettsia conorii* based on multi-locus sequence typing and an emended description of *Rickettsia conorii*. *BMC Microbiol.* 5:11. <http://dx.doi.org/10.1186/1471-2180-5-11>.
 32. Harrus S, Perlman-Avrahami A, Mumcuoglu KY, Morick D, Baneth G. 2011. Molecular detection of *Rickettsia massiliae*, *Rickettsia sibirica mongolitimonae* and *Rickettsia conorii israelensis* in ticks from Israel. *Clin. Microbiol. Infect.* 17:176–180. <http://dx.doi.org/10.1111/j.1469-0691.2010.03224.x>.
 33. De Sousa R, Santos-Silva M, Santos AS, Barros SC, Torgal J, Walker DH, Bacellar F. 2007. *Rickettsia conorii* Israeli tick typhus strain isolated from *Rhipicephalus sanguineus* ticks in Portugal. *Vector Borne Zoonotic Dis.* 7:444–447. <http://dx.doi.org/10.1089/vbz.2006.0628>.
 34. Ereemeeva ME, Bosserman EA, Demma LJ, Zambrano ML, Blau DM, Dasch GA. 2006. Isolation and identification of *Rickettsia massiliae* from *Rhipicephalus sanguineus* ticks collected in Arizona. *Appl. Environ. Microbiol.* 72:5569–5577. <http://dx.doi.org/10.1128/AEM.00122-06>.
 35. Beati L, Roux V, Ortuño A, Castella J, Porta FS, Raoult D. 1996. Phenotypic and genotypic characterization of spotted fever group *Rickettsia* isolated from Catalan *Rhipicephalus sanguineus* ticks. *J. Clin. Microbiol.* 34:2688–2694.
 36. Bitam I, Parola P, De La Cruz KD, Matsumoto K, Baziz B, Rolain J-M, Belkaid M, Raoult D. 2006. First molecular detection of *Rickettsia felis* in fleas from Algeria. *Am. J. Trop. Med. Hyg.* 74:532–535.
 37. Boudebouch N, Sarih M, Beaucournu J-C, Amarouch H, Hassar M, Raoult D, Parola P. 2011. *Bartonella clarridgeiae*, *B. henselae* and *Rickettsia felis* in fleas from Morocco. *Ann. Trop. Med. Parasitol.* 105:493–498. <http://dx.doi.org/10.1179/1364859411Y.0000000038>.
 38. Capelli G, Montarsi F, Porcellato E, Maioli G, Furnari C, Rinaldi L, Oliva G, Otranto D. 2009. Occurrence of *Rickettsia felis* in dog and cat fleas (*Ctenocephalides felis*) from Italy. *Parasites Vectors* 2(Suppl 1):S8. <http://dx.doi.org/10.1186/1756-3305-2-S1-S8>.
 39. Ereemeeva ME, Karpathy SE, Krueger L, Hayes EK, Williams AM, Zaldivar Y, Bennett S, Cummings R, Tilzer A, Velten RK, Kerr N, Dasch GA, Hu R. 2012. Two pathogens and one disease: detection and identification of flea-borne rickettsiae in areas endemic for murine typhus in California. *J. Med. Entomol.* 49:1485–1494. <http://dx.doi.org/10.1603/ME11291>.
 40. Parola P, Davoust B, Raoult D. 2005. Tick- and flea-borne rickettsial emerging zoonoses. *Vet. Res.* 36:469–492. <http://dx.doi.org/10.1051/vetres:2005004>.