ORIGINAL ARTICLE

The role of *Rhipicephalus sanguineus* ticks parasitizing dogs in the spread of tick-borne rickettsial pathogens in the city of Sevastopol

E. E. Alieva[1], E. I. Bondarenko[2], K. D. Maliy[3], A. N. Shvalov[4], E. A. Verbenets[5] and M. T. Gafarova[3]

1) Federal State Budgetary Institution 'N.I. Pirogov Saki Military Clinical Sanatorium', Ministry of Defence of Russia, Saki, 2) AO 'Vector-Best', Novosibirsk, 3) S.I. Georgievsky Medical Academy (Academic Unit), V.I. Vernadsky Crimean Federal University, Simferopol, 4) State Research Centre of Virology and Biotechnology 'Vector', Koltsovo, Novosibirsk and 5) Centre for Prevention and Control of HIV/AIDS, Sevastopol, Crimea, Russia

Abstract

The occurrence of Mediterranean fever with periods of increase and decrease has been recorded in the Crimean peninsula. The city of Sevastopol and its vicinity are known endemic areas for this disease. Some of the most active agents in the spread of this rickettsiosis are feral and abandoned dogs. The aim of this study was to test ticks parasitizing dogs in Sevastopol for the presence of *Rickettsia* using molecular methods. The testing of ticks was carried out using real-time PCR and the 'Real Best DNA Rickettsia species' kit (AO 'Vector-Best') followed by sequence identification of the rickettsial DNA detected. The DNA marker for *Rickettsia* species (a conservative area of citrate synthase gene, *glt*A) was detected in 16 of 84 (19.1%) samples of *Rhipicephalus sanguineus* ticks tested. Larger fragments of *glt*A, *omp*A and *sca*4 were amplified and sequenced for 10 of 16 PCR-positive samples. *Rickettsia* DNA amplified from eight of the samples matched the sequence of *Rickettsia conorii conorii* Malish, the causative agent of Mediterranean fever. The sequences of *Rickettsia* DNA from two other ticks had the closest match to homologous fragments of *Rickettsia massiliae*, a pathogenic spotted fever rickettsia that was identified in the Crimean Peninsula for the first time as part of this study. The detection of two pathogenic species of *Rickettsia* in the studied ticks suggests the potential for two rickettsial diseases in the region and warrants further epidemiological and clinical studies.
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Corresponding author: M.T. Gafarova, S.I. Georgievsky Medical Academy (Academic Unit), V.I. Vernadsky Crimean Federal University, Boulevard Lenina, 5/7, Simferopol, Crimea, Russia.
**E-mail:** muniver@mail.ru

Introduction

The first cases of Mediterranean spotted (Marseilles) fever on the Crimean Peninsula were identified and described in the city of Sevastopol in 1936 by A.Ya. Alymov [1]. Subsequently, similar cases were identified in Yalta (1937–1938), Yevpatoria and Kerch (1938) [2]. Kulagin established the epidemiological connection of Mediterranean spotted fever disease in Crimea with tick bites and contact with dogs [2]. The role of the brown dog tick, *Rhipicephalus sanguineus* in transmission of *Rickettsia* was first established by Caminopetros and Brumpt in the 1930s [3,4]. In the 1960s Klyushkina corroborated these observations and demonstrated involvement of *Rh. Sanguineus* in the existence of natural and anthropogenic foci of Mediterranean fever in Crimea [5]. It is established that *Rh. Sanguineus* is not only a carrier, but also a reservoir of the causative agent of infection—*Rickettsia conorii* [5]. Furthermore, it was determined that the Crimean focus of Mediterranean spotted fever has the same environmental and epidemiological characteristics as endemic foci in the Mediterranean [6,7].

The brown dog tick *Rhipicephalus sanguineus* (Latreille, 1806) is a specialized dog parasite that is common on all continents and in countries with tropical, subtropical and temperate climates [8]. On the Crimean Peninsula *Rh. Sanguineus* has a ubiquitous distribution. Dogs are the main hosts for *Rh. Sanguineus*, but in cases of severe infestation, this tick can also be
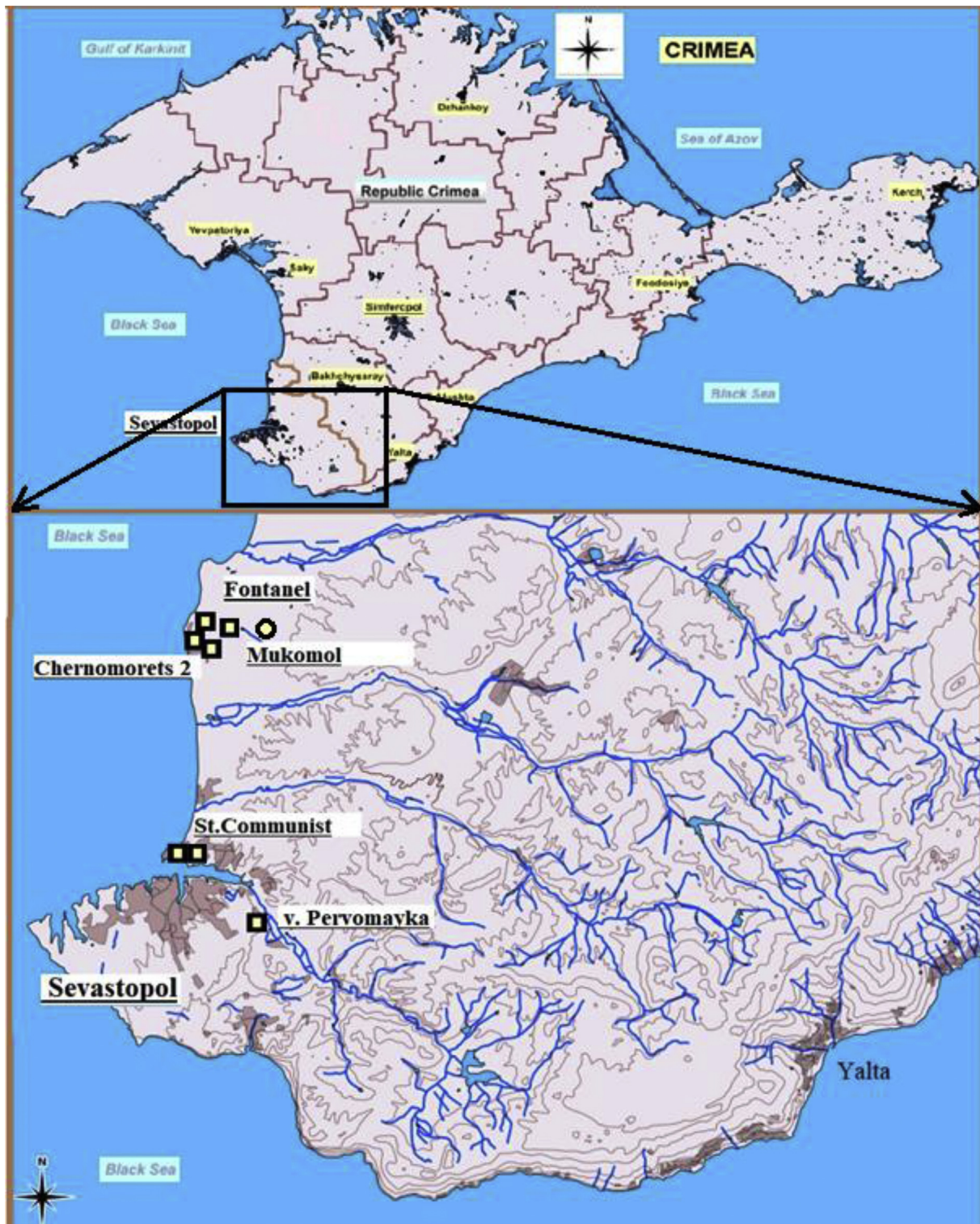
FIG. 1. Map of *Rhipicephalus sanguineus* collection sites. Designations: ☐squares represent dogs and the ⊙circle represents the cat.

found on cows, sheep, goats and cats [5]. Furthermore, birds, particularly field, steppe and crested larks, partridge and quail, are possible hosts for the larval and nymphal stages of ticks [9].

Rhipicephalus sanguineus accounted for 26.9% of the more than 125 000 ticks belonging to 18 species collected in the Crimea over 20 years from 1981 to 2001 [9]. A 1993 outbreak of adults

of *Rh. Sanguineus* on the Kerch Peninsula was also associated with an increase in the number of stray dogs and favourable weather conditions conducive to the survival, development and reduction in the development time of larvae and nymphs, and the numbers of eggs [10].

Resurgence of Mediterranean spotted fever has been described over the years in Crimea. A detailed study of domestic foci and an analysis of the incidence of Marseilles fever in Sevastopol are summarized in the works of Pakshin and his group [11]. A high incidence rate was observed after World War II (1947–1949), when the infection (or prevalence) rate was 7.0, 11.6 and 12.5 cases per 100 000 people in 1947, 1948 and 1949, respectively. Since 1991, another increase in incidence was observed with a peak of 13.8 cases per 100 000 people in 1996 [12]. This latest increase in morbidity correlated with an increase of stray dogs and the number of *Rh. Sanguineus* [11]. In 1996, an outbreak of Mediterranean spotted fever was recorded in the Saki region: 26 cases including one fatality were identified [2]. Subsequently, cases of Marseilles fever were identified in the Black Sea, Bakhchisaray, Simferopol and Leninsky districts, including cases recorded in the cities of Yevpatoria, Simferopol, Yalta, Feodosia and Kerch [2]. Currently, according to the City Infectious Diseases Hospital, cases of Mediterranean (Marseilles) spotted fever are continuously reported in Sevastopol and the incidence rate exceeds the incidence rate in Crimea [13]. Favourable climatic conditions for the development of *Rh. Sanguineus* and regularly recorded disease cases firmly established Sevastopol's status as an active endemic region for Mediterranean spotted fever [5,14,15]. In the 1980s, a strain of *R. conorii* identical to the *R. conorii* strain M-1 was isolated in Sevastopol [16].

The purpose of this study was to analyse ticks parasitizing dogs in the city of Sevastopol to detect infection with rickettsia and to identify them using molecular genetic methods.

Materials and methods

The collection of ticks was carried out in the city of Sevastopol; the map shows the places where ticks were collected (Fig. 1).

Ticks were removed from animals in September 2016 from ex-urban subdivisions of the Balaklava (v. Pervomayka), Leninsky (st. Communist) and Nakhimovsky (Fontanel, Mukomol, Chernomorets-2) districts of the city of Sevastopol, in places with large numbers of guard dogs, dogs used for protecting livestock and stray dogs.

Eight animals were examined: seven dogs and one cat. Eighty-four tick specimens were collected, including 17 males, 12 females and 55 nymphs. All ticks were identified as *Rh. Sanguineus* according to the morphological taxonomic keys described by N.A. Filippova [17].

Extraction and testing of tick DNA

Each tick specimen was individually washed in 300 μL of 96% ethanol, followed by ethanol removal using a vacuum aspirator. Residual ethanol was removed by rinsing ticks in 500 μL of physiological saline. Individual ticks were homogenized using a MagNaLyser device (Roche Diagnostics, Basel, Switzerland) using the Matrix-K sample grinding kit (AO 'Vector-Best', Novosibirsk, Russia), which uses test tubes containing small ceramic balls, as described elsewhere [18]. Homogenization was carried out by shaking the tubes containing ticks on a MagNaLyser for 1.5 minutes at a speed of 7000 rpm. To obtain tick suspensions, 350 μL of sample preparation solution (Vector-Best JSC Novosibirsk) was added to each vial containing tick homogenate. Total DNA was extracted using the 'RealBest Extraction 100' reagent kit according to the manufacturer's instructions (AO 'Vector-Best'), using 100 μL of each tick suspension individually; the remaining samples were frozen and stored at −70°C. Purified DNA was eluted using 300 μL of the elution buffer included in the isolation kit.

Prepared tick DNA was tested for rickettsia DNA marker (conserved region of the *glt*A gene) using real-time PCR using the commercial reagent kit 'RealBest DNA Rickettsia species' (AO 'Vector-Best'), which is based on a hybridization DNA probe specific to a selected region of rickettsia DNA. For the real-time PCR, 50 μL of tick DNA was added to the reaction vial containing lyophilized (freeze-dried) reaction mixture containing 0.5 μM of each primer and 0.25 μM specific probe. Homologous *glt*A fragment of *Rickettsia sibirica sibirica* isolated from a tick and cloned into a plasmid was used as a positive control sample, which is part of the 'RealBest DNA of Rickettsia species' kit. Two negative controls were processed similarly to the tick DNA samples and included in each reaction; negative control #1 was a cattle serum included in the 'RealBest Extraction 100' kit and negative control #2 was a tick suspension that had previously tested negative for the DNA of *Rickettsia* species. Real-time PCR was performed using a CFX96 thermocycler (Bio-Rad, 1000 Alfred Nobel Drive, Hercules, CA 94547, USA).

Design of primers, sequencing and species identification of Rickettsia

To complete the species identification of *Rickettsia* in tick DNA samples, fragments of *glt*A, *omp*A and *sca*4 genes were amplified and sequenced using primers listed in Table 1. The primer design and annealing temperature of all primers used for PCR amplification of individual gene fragments were carried out using Integrated DNA Technologies software (IDT, https://eu.idtdna.com). Search for known nucleotide sequences was carried out in the NCBI database (http://www.ncbi.nlm.nih.gov/genbank). When designing oligonucleotide primers, the

TABLE 1. Oligonucleotide primers used for amplification and sequencing of *Rickettsia* genes

Target gene	Primer name	Primer sequence 5′–3′	Primer position and amplicon length (nt)	Reference sequence	GenBank Accession no.
Citrate synthase: *glt*A	RS-F1 RS-R1	GCAAGTATTGGTGAGGATGTA GTTCAGGGTCTTCGTGCA	62–1214, 1153	*R.conorii* Seven	U59730
Outer membrane protein A: *omp*A	RSp-F14 RSp-R2	GCGATAATGCTGAGTAGTAGC GCAACAAGTTACCTCCCGTTA	61–376, 316	*R. conorii* URRCMFEe29	AY346453
Cell-surface antigen: *sca*4	R.Sca4-F3 R.Sca4-R1	GCAGATGTTAGAAAAGGCAGTA TCCGCTGATGCCATAATAAGT	1599–2174, 576	*R. conorii* Malish 7	AE006914

following requirements were taken into account: the absence of extended regions of repeating nucleotides, the absence of complementary sequences longer than three bases inside the oligonucleotides and a high content of GC bases (at least 50%). The probability of the formation of secondary structures by oligonucleotides, as well as homodimers and heterodimers, reducing the effectiveness of PCR was analysed for each primer set designed. Sequence alignments were done using the Vector NTI Advance v. 11.5.5 (Life Technologies, 5791 Van Allen Way, 92008 Carlsbad, CA, USA). The synthesis of primers was carried out in the laboratory of chemical synthesis of AO 'Vector-Best'.

To amplify each of three fragments of *Rickettsia* DNA, 45 μL of the total tick DNA and 0.5 μM of each forward and reverse primers was used with *Taq* polymerase. The amplification programme used consisted of the following steps: Step 1, 94°C for 1 min; Step 2, five cycles at 94°C for 15 seconds, 62°C for 20 seconds and 72°C for 20 seconds; Step 3, 45 cycles at 94°C for 15 seconds, 60°C at 30 seconds and 72°C for 30 seconds. All reactions included negative control (sterile water) and positive recombinant control plasmid (DNA fragment of *R. sibirica sibirica*) for each of the three target gene fragments.

Sequencing was performed by the Sanger method using an ABI Prism 3100 Genetic Analyzer sequencer (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA, 94404, USA) at the SANAN Genomika Collective Use Center (Novosibirsk, Russia). Sequenced fragments were compared with the homologous nucleotide sequences of *Rickettsia* available through the NCBI database using BLASTn. The nucleotide sequences of the fragments were deposited in the NCBI database under the following numbers: *R. conorii conorii*—KY640399 (isolate Crimea-2016-1, *glt*A) and KY640402 (isolate Crimea-2016-2, *glt*A), KY640400 (isolate Crimea-2016-1, *omp*A) and KY640403 (isolate Crimea-2016-2, *omp*A), KY640401 (isolate Crimea-2016-1, *sca*4) and KY640404 (isolate Crimea-2016-2, *sca*4); *Rickettsia massiliae*—KY640405 (isolate Crimea-2016-3, *glt*A) and KY640408 (isolate Crimea-2016-4, *glt*A), KY640406 (isolate Crimea-2016-3, *omp*A) and KY640409 (isolate Crimea-2016-4, *omp*A), KY640407 (isolate Crimea-2016-3, *sca*4) and KY640410 (isolate Crimea-2016-4, *sca*4).

Homologous sequences were aligned using the Unipro UGENE 1.31 toolkit [19] and the MAFFT algorithm [20]. Phylogenetic analysis was performed using the PhyML maximum likelihood method [21] using the three-parameter model T92 [22] with the search for parameter 1 (proportion permanent sites), and system requirements tree (SRT) and nearest neighbour interchange tree optimization type. Support indices were determined by bootstrap method with 1000 repetitions.

Results

As a result of inspection of eight captured animals (seven dogs and one cat), 84 ticks were collected. Ticks were found on all animals. The largest number of ticks was removed from dogs nos 2, 6 and 7 from the Leninskyi and Nakhimovskyi districts; more than 70% of the studied ticks were collected from these three animals. All ticks were identified as *Rh. Sanguineus*.

A conserved region of the *glt*A gene, universal *Rickettsia* DNA marker, was detected using real-time PCR in 16 of 84 (19.1%) DNA samples isolated from individual ticks. Ten *Rickettsia*-positive samples were further characterized by sequencing *glt*A, *omp*A and *sca*4 fragments (Table 2).

Nine nymphs and seven adult ticks tested positive for the *glt*A *Rickettsia* marker using real-time PCR. These ticks were removed from three (in the Nakhimovsky district) of the eight animals examined. Five ticks were removed from dog no. 5, only one nymphal tick tested positive for *glt*A *Rickettsia* marker. Ten of the 33 ticks removed from dog no. 6 tested positive for *glt*A *Rickettsia* marker (30.3%), and 5 of the 16 ticks removed from dog no. 7 were also positive (31.3%).

To identify the *Rickettsia* species DNA in tick samples testing positive by real-time PCR, three other gene fragments were amplified: *glt*A, *omp*A and *sca*4. Ten of 16 (62.5%) tick DNA samples that contained the *Rickettsia glt*A marker yielded all three gene fragments, which were sequenced. These positive DNA samples had low Ct values (18–32 cycles) when tested using the *Rickettsia* species RealBest DNA reagent kit (Table 2). Six other positive samples had higher Ct values (35–37 cycles)

and did not yield enough DNA for sequencing because of the low amount of rickettsial DNA estimated to be less than 50 genome equivalents per reaction in a volume of 50 μL. The1064-bp fragment of *glt*A, 277-bp fragment of *omp*A and 539-bp fragment sca4 were sequenced for each of ten samples. The obtained sequences for all three genes belonged to two groups. The first group was from eight ticks; these sequences were 100% identical to each other and exhibited a 100% match to the homologous sequences of *R. conorii conorii str.* Malish 7 (AE006914. All sequenced fragments (*glt*A, *omp*A and *sca*4) of isolates Crimea-2016-1 and Crimea-2016-2 identified as *R. conorii conorii* sequences fully matched homologous sequences of strain Malish 7 (GenBank Accession nos AE006914), and belong to the same cluster of sequences on each phylogenetic tree analyzed (Figs. 2 and 3).

The second group of identical sequences was obtained from two ticks (Crimea-2016-3, Crimea-2016-4) and corresponded to those of *R. massiliae* (str. AZT80 (CP003319). The *glt*A fragment sequences of the Crimea-2016-3 and Crimea-2016-4 isolates were identical to *Candidatus Rickettsia kulagini* strain Kertch (GenBank Accession no. DQ365806) and exhibited 99.7% (1054/1057 nucleotides) homology to *glt*A sequences of *R. massiliae* AZT80 (CP003319) and MTU5 (CP000683) (Fig. 2). The *sca*4 fragment of the same Crimean isolates had 99.3% (535/539 nucleotides) sequence similarity with strain AZT80 (CP003319) (Fig. 3), whereas the 274-bp *omp*A fragment was 100% identical with *omp*A gene sequences from the *R. massiliae* NMG-70 (MH549236) and JMR44 (KY440234) isolates. Furthermore, it was determined, that ticks infesting dog no. 5 were positive for the DNA of *R. conorii conorii* whereas dogs no. 6 and no. 7 were both infested with different ticks carrying DNA of two different pathogens, *R. conorii conorii* and *R. massiliae* (Table 2).

The *glt*A and *sca*4 fragment sequences of isolates Crimea-2016-3 and Crimea-2016-4 belong to clades (support indices 64 and 96, respectively) closely related to *R. massiliae* and according to the *omp*A gene, they are identical to several *R. massiliae* sequences. The *glt*A fragment sequences of the Crimea-2016-3 and Crimea-2016-4 isolates and *Candidatus*

R. kulagini strain *Kertch* form a unique lineage closely related to homologous sequences of other *R. massiliae* strains isolated worldwide (Fig. 2). However, our data are insufficient to unambiguously assign the isolates Crimea-2016-3 and Crimea-2016-4 to a new species, therefore, we attributed them to the existing *R. massiliae*.

Discussion

Molecular genetic methods have been introduced and received broad application for laboratory diagnosis of rickettsial agents, so replacing traditional serological methods. Such approaches have led to the identification and description of several new species of *Rickettsia* in Russia [23–27]. A recent study reported detection of several pathogenic species of rickettsiae, including *Rickettsia raoultii*, *Rickettsia monacensis* and *Rickettsia aeschlimannii* in ticks from Crimea [26]. However, for several decades, Mediterranean spotted fever was the only formally reported tick-borne rickettsiosis in Crimea, and *R. conorii* was considered the only circulating pathogenic *Rickettsia*. As a rule, patient diagnosis is made either based on clinical manifestations (the presence of a characteristic triad: eschar, rash and regional lymphadenitis) or according to epidemiological data (history of tick attachment or tick removal).

Our testing of *Rh. Sanguineus* collected in Sevastopol resulted in the identification of DNA of *R. conorii conorii* and *R. massiliae*. When analysing the results obtained, it is necessary to take into account the possibility of horizontal transmission of rickettsia from infected ticks to non-infected ticks during co-feeding on animals (dogs), acquisition of rickettsiae with blood from rickettsiemic animals, as well as the contact of animals with each other [27]. That is critical as ticks can become infected with rickettsia by direct spread of bacteria from an infected tick to an uninfected tick during feeding and being in close proximity to each other on the same host [28]. The mechanism of transmission of tick-borne pathogens through co-feeding is well described in the case of transmission of *R. conorii israelensis* between *Rh. Sanguineus* [29]. Therefore, the estimates of true

TABLE 2. The results of PCR testing of suspensions of *Rhipicephalus sanguineus* for the presence of the *Rickettsia* DNA marker and subsequent identification of *Rickettsia* species by sequencing

Location, district	Animal species and ID number	No. Of ticks collected	No. Of ticks tested positive for *glt*A marker and their life stage	Number of ticks tested positive for three sequence fragments (*glt*A, *omp*A, sca4)	*Rickettsia* species identified (number)
Balaklava	Dog (#1)	6	0	ND*	NA*
Leninskyi	Dog (#2, #4)	17	0	ND	NA
Nakchimovskyi	Dog (#3, #5, #6, #7)	57	16 (7 adults and 9 nymphs)	10	*R. conorii conorii* (8)
	Cat (#1)	4	0	ND	*R. massiliae* (2)
					NA

Abbreviations: NA, not applicable; ND, none detected.
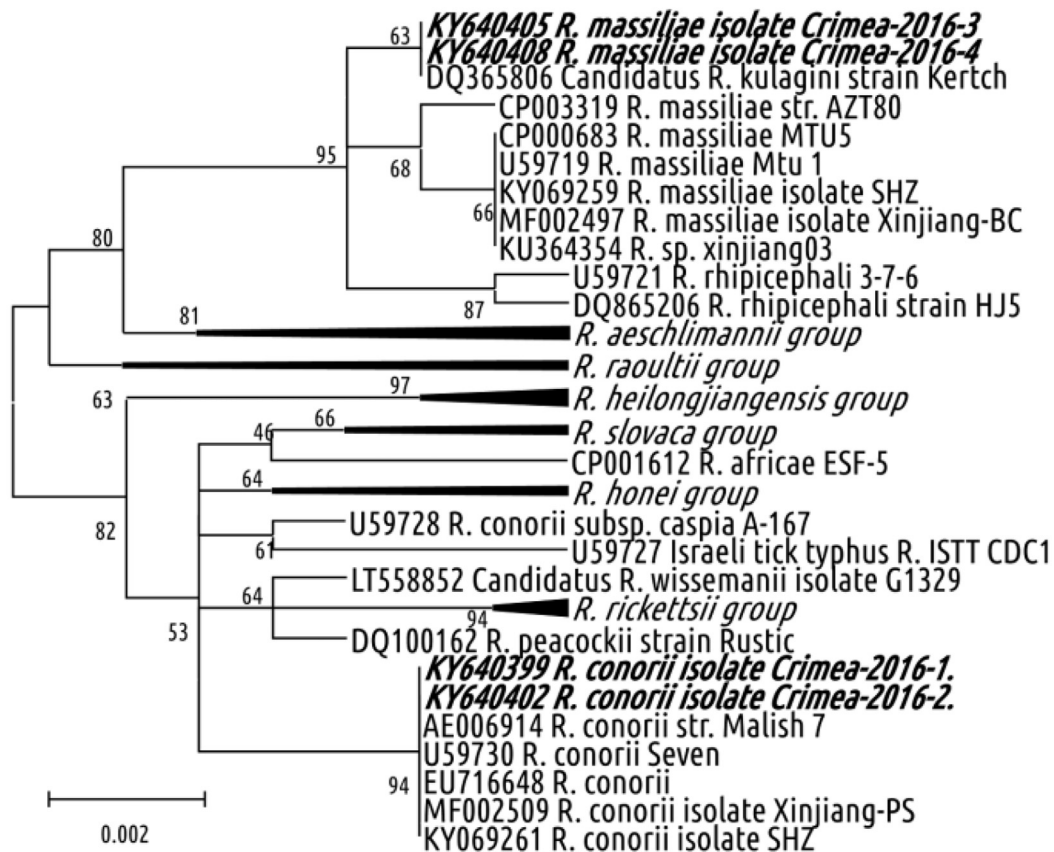
FIG. 2. Phylogenetic analysis of *glt*A gene fragments from 2016 Crimean isolates of *Rickettsia*.

prevalence of *Rickettsia* in tick specimens tested positive for *Rickettsia* DNA markers may not be entirely accurate because of the constant contact of animals with each other, the possible exchange of ticks and, accordingly, native tick rickettsia acquisition both from infected ticks and from an infected animal host.

The results of our study confirm the hypothesis that the brown dog tick *Rh. Sanguineus* serves as a common vector of the Mediterranean spotted fever agent *R. conorii conorii* in the region. Furthermore, our study corroborated tick circulation of another spotted fever group rickettsiae related to *R. massiliae* in *Rh. Sanguineus* from Crimea, and reported additional genetic characteristics of these DNAs. The aetiological role of *R. massiliae* was first established in a retrospective analysis of the *glt*A and *omp*A fragment sequences of a rickettsia strain isolated from the patient's blood in 1985 in Sicily [30]. In 1990, *R. massiliae* was first isolated from *Rh. Turanicus* collected from a horse in France [31]. In 1993, *R. massiliae* received the official status of species [32]. *Rickettsia massiliae* has also been found in *Rh. Sanguineus* parasitizing dogs in southern California and Arizona [33]. DNA of *R. massiliae* was detected in different species of ticks of the genus *Rhipicephalus*: *Rhipicephalus senegalensis*, *Rhipicephalus sulcatus*, *Rhipicephalus lunulatus*,

Rhipicephalus mushamae, *Rh. Sanguineus* and *Rhipicephalus turanicus* [6]. Diversity of *R. massiliae* and closely related agents is not fully understood. The clinical symptoms caused by *R. massiliae* are very similar to the symptoms of the disease caused by *R. conorii* [7,34]; however, there are not enough data and diagnostic possibilities to establish the epidemiological role of this rickettsia in Crimea. However, the presence of this *Rickettsia* in brown dog ticks and a high rate of dog infestation serves as a reliable indicator, suggesting that *R. massiliae* infections may occur, but are not diagnosed in Crimea [7,33,35−37].

In general, laboratory diagnosis of rickettsioses is difficult because of the non-specific clinical symptomatology, the lack of clear serological indicators during the acute stage of illness and the limited availability of molecular methods [27]. Moreover, there is a low awareness of rickettsioses among practicing physicians, and it may take a long time before a patient is referred to the infectious disease doctor, or diagnosis does not occur in many instances. Until recently, diagnostic capabilities were limited to serological testing of patients' sera in the complement fixation test using commercial tests, which are not manufactured anymore. Complement fixation antibodies
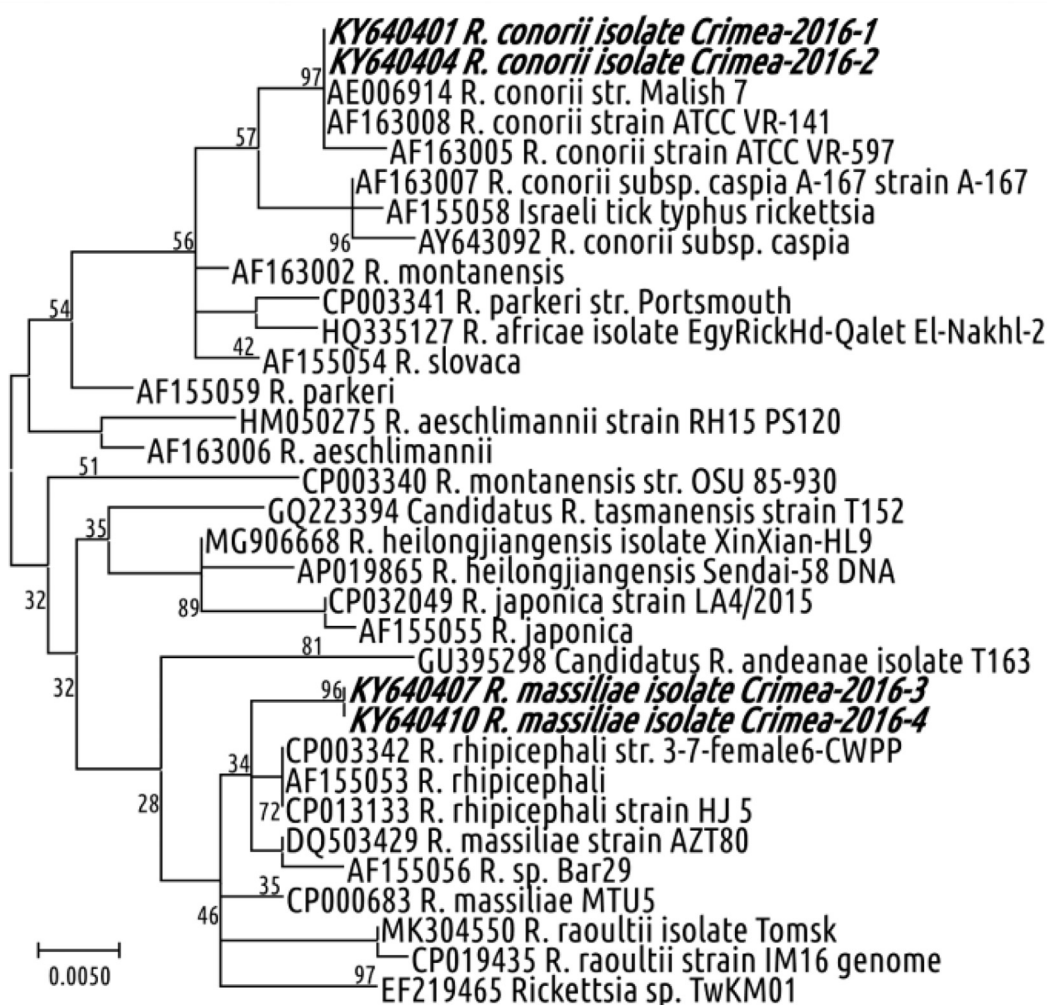
FIG. 3. Phylogenetic analysis of fragments of the *sca*4 gene fragments from 2016 Crimean isolates of *Rickettsia*.

reacting to *R. sibirica* antigen were detected in 2.2% of healthy individuals from Sevastopol tested by Kulagin in 1960 [38]. A similar study conducted in 2009 by Verbenets reported positivity of 11.1% of the serum samples tested [13]. Many of the people tested denied having the disease or experiencing its characteristic symptoms [13]. These observations indicate possible undiagnosed disease, a higher rate of human infection or the occurrence of mild, atypical clinical forms of illness due to exposure to ticks infected with less pathogenic or avirulent rickettsia [39].

The obtained results indicate the need for further detailed study of rickettsioses of the spotted fever group and the genetic diversity of rickettsia circulating in the Crimea. The use of molecular biological methods of laboratory diagnostics opens up the possibility of faster and more efficient identification of rickettsioses and improvement of the epidemiological surveillance system [40].

Conflict of interest

There is no conflict of interests.

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