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Molecular Detection of *Rickettsia* spp. in Free Living Ticks in Military Instruction Areas in Southeast Brazil

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HIGHLIGHTS

- The first molecular detection of *Rickettsia* spp. in military instruction areas.
- Of the 1,885 tick samples tested, 11 were amplified for the *glt*A gene of *Rickettsia*.
- Two positives *Rickettsia* with phylogenetic relationships to pathogenic rickettsiae.

Abstract: The present study aimed for the molecular detection of *Rickettsia* spp*.* in ticks collected from military training areas in southeastern Brazil. Previously, a total of 9,374 free-living ticks in 66 military instruction areas of five Military Organizations (MOs) were collected. From these, a sample of 1,885 ticks was used for molecular analysis..DNA extraction was performed using the “HotSHOT” technique, and PCR was performed using the *Rickettsia* spp*. glt*A, *17kDa* and *omp*A genes. It was found that 11 samples produced DNA sequences of sizes compatible with the *Rickettsia* spp*. gltA* and *17kDa* gene and two of these, also for *omp*A, with a prevalence of 0.58% (11/1885). Analyses of amplified fragments of *glt*A identified the samples as *Rickettsia bellii* in seven nymphs of *Amblyomma dubitatum* and one nymph of *Amblyomma sculptum*, and identified samples as *Rickettsia* sp*.* in two nymphs of *A. dubitatum* and one of *A. sculptum.* This finding warns the possibility of the risk of exposure of military personnel to ticks infected with *Rickettsia* spp*.* during training.

Keywords: Zoonosis; PCR; soldier; tick-borne diseases; occupational risk.

INTRODUCTION

Military activities can enhance the emergence and transmission of infectious diseases, exposing troops to diseases caused by agents transmitted by vectors such as mosquitoes and ticks [1-3]. Ticks are the main vectors of agents that cause occupational zoonotic diseases [4,5], and are among the two main groups of occupational biological risks [6].

In recent years, the increase in incidence, emergence of diseases transmitted by ticks, and geographic expansion of these vectors have attracted the attention of public health authorities [7]. Bacteria from the Rickettsiaceae family are the most frequently emerging etiological agents transmitted by ticks [8].

Tick-borne rickettsiae are widely distributed and transmitted mainly by different species of ixodids in the Americas, Europe, Asia, Africa, and Oceania [9]. In this way, rickettsiosis has to have represented a serious public health problem for military personnel worldwide [10-12] and is for are among the main health issues that the Brazilian military may be subjected to during peacekeeping missions [13].

In Brazil, the most frequent rickettsiosis infecting humans has been caused by *Rickettsia rickettsii* and *Rickettsia parkeri* strain Atlantic Forest (spotted fever; SF group) in different epidemiological scenarios involving *Amblyomma sculptum*, *Amblyomma aureolatum*, and *Amblyomma ovale* ticks [14-16]. Such ticks have a wide geographical distribution in our country [15], with the possibility of rickettsiosis affecting military personnel in training or operations, with negative impacts on the health of troops, as has occurred in other armed forces [10,17-19]. Although Rocky Mountain SF caused by *R. rickettsii* is one of the most severe rickettsioses and has been affecting military personnel in training in the Americas for some time [10], *R. parkeri* appears to be a recent threat, as it is an emerging disease that has been identified in several regions across the American continent, representing a serious public health problem due to the existence of conditions for its wide distribution [20,21].

Despite the constant training and operations carried out by the Brazilian Armed Forces, allowing the military to be more exposed to vectors [13], few studies have analyzed the presence of rickettsiae in ticks from military training areas. Thus, this study aimed to search, through molecular biology techniques, *Rickettsia* spp. in ticks collected from the environment in military training areas in southeastern Brazil.

MATERIAL AND METHODS

Ethical and legal aspects of scientific research

This study was authorized and approved by the Research Ethics and Governance Committee of the Academia Militar das Agulhas Negras, through the Minutes of the Meeting of the Research Ethics and Governance Committee in AMAN No. 001/2019, and carried out after authorization for the transport of biological samples by the Biodiversity Authorization and Information System (SISBio), under No. 68991-1.

Tick sampling

The research was conducted on tick samples collected in the free-living phase in military training areas of five Military Organizations (MOs) located in five municipalities in the southeastern region of Brazil (Figure 1). The MOs sampled are located in the cities of Três Corações – Minas Gerais (Escola de Sargento das Armas - ESA), Juiz de Fora - Minas Gerais (Campo de Instrução de Juiz de Fora and Centro de Educação Ambiental e Cultura - CIJF/CEAC), Paracambi – Rio de Janeiro (Depósito Central de Munições - DCMun), Resende - Rio de Janeiro Academia Militar das Agulhas Negras - AMAN) and Campinas – São Paulo (11th Brigada de Infantaria Leve - 11th Bda Inf L and Escola Preparatória de Cadetes do Exército - EsPCEx). The cities of Juiz de Fora, state of Minas Gerais (MG) and, Paracambi and Resende in the state of Rio de Janeiro are located in an area of the Atlantic Forest biome, while Três Corações-MG and Campinas, state of São Paulo are located in Cerrado biome. The geographic coordinates, complete characterization of the collected areas and, morphological identification of ticks were described by Prado and coauthors [22]. A survey was conducted in 2019 between August and November, the months with the highest incidence of Brazilian spotted fever (BSF) [35]. In total, 9,374 free-living ticks in 66 military instruction areas of the MO were collected. From these, a sample of ticks was used for molecular analysis.

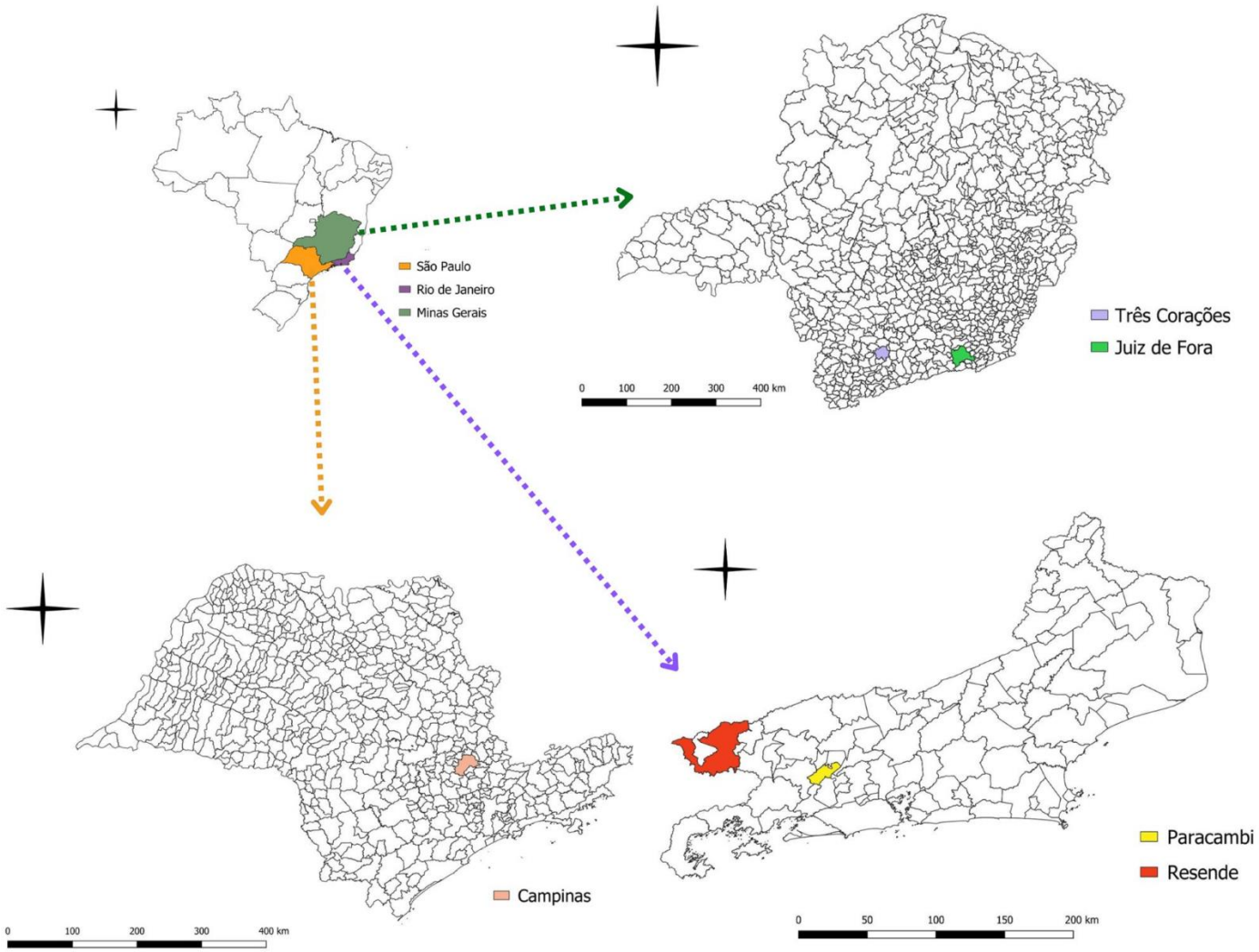

Figure 1. Study area maps: Brazil, with emphasis on the state of São Paulo, Minas Gerais e Rio de Janeiro in the southerneast region; State of São Paulo, highlighting the municipality of Campinas; State of Minas Gerais, highlighting the municipality of Três Corações and Juiz de Fora. State of Rio de Janeiro, highlighting the municipality of Resende and Paracambi.

This sample size was calculated based on the estimated prevalence of 50% of ticks infected by rickettsia in the study regions, given the lack of previous similar research in military areas, according to the formula proposed by Agranonik & Hirakata [23]. Considering a margin of error of 6% and confidence level of 95%, a minimum sample of 267 ticks per MO was calculated.

From the minimum number of ticks to be analyzed for each MO, a stratified sample was calculated and taken from each of the 66 instruction areas surveyed in the MO, proportional to the number of ticks collected from each genus and/or species and stage of life in relation to the total number of ticks collected in the areas of each MO.

The larvae captured in clusters were considered as only one individual. For sample calculation purposes, a pool of five larvae was taken from each of these collected clusters, from which the DNA was extracted together and subsequently tested. Furthermore, all adult ticks, nymphs of different species, except *A. sculptum* and, larvae of *Dermacentor nitens* and *Rhipicephalus* sp., regardless of the sample quantity calculated, were purposely included in the sample to be tested, with the aim of researching *Rickettsia* spp. in these different genera and species and increasing the final number of ticks to be tested in each MO.

Thus, a stratified random sample of 1,494 ticks collected from 66 areas of military instruction was initially selected for molecular analysis. Additionally, due to the absence of positives, another 391 ticks from DCMun, AMAN, and 11ª Bda InfL/EsPCEx were inserted, totaling 1,885 ticks tested, as shown in Table 1.

Table 1. Number of tick, by stages of development and species, from MO in the Southeast region of Brazil used for molecular research of *Rickettsia* spp. in this study.

Species/Stage	Number of tick samples tested by MO					
	ESA	**CIJF**	**DCMun**	**AMAN**	**11ª Bda Inf L/EsPCEx**	**Total**
Amblyomma sp. larvae	32	43	0	44	35	154
Amblyomma sp. larvae pool	0	0	4	13	24	41
Amblyomma sculptum – nymph	238	200	299	374	378	1489
Amblyomma sculptum – adult	7	0	2	8	20	37
Amblyomma dubitatum – nymph	3	28	0	3	1	35
Amblyomma dubitatum – adult	0	1	0	3	9	13
Amblyomma brasiliense – nymph	0	0	0	42	0	42
Amblyomma brasiliense – adult	0	0	0	1	0	1
Amblyomma longirostre – nymph	0	0	0	2	0	2
Amblyomma aureolatum – nymph	2	0	0	0	0	2
Dermacentor nitens – larvae	0	0	0	7	0	7
Dermacentor nitens – larvae pool	0	0	0	26	0	26
*Rhipicephalu*s sp. – larvae	18	0	0	0	0	18
Ixodes sp. – larvae	0	5	0	0	0	5
Haemaphysalis sp. – larvae	0	13	0	0	0	13
Total	300	290	305	523	467	1885

MO = Military Organization; **ESA** = School of Sergeants of Arms; **CIJF/CEAC** = Field of Instruction of Juiz de Fora/Center for Environmental Education and Culture; **DCmun** = Central Ammunition Depot; **AMAN** = Agulhas Negras Military Academy; **ESPCEx** = Preparatory School for Army Cadets; **11ª Bda Bda Inf L** = 11ª Light Infantry Brigade.

Differences in the number of tick tested in each of the MOs were due to mathematical rounding that occurred in the calculation of the stratified sample, where all results below 01 (one) were rounded to that value, and to the insertion of all ticks of different species except *A. sculptum* in molecular research.

Molecular analyses

DNA extraction from ticks was performed using the "HotSHOT" method as described by Truett and coauthors [64]. For quality control of the DNA extraction technique, some samples (10%) of each extraction battery were submitted for prior amplification of the partial sequence of the tick 16S rRNA gene, following the protocol of Mangold and coauthors [24].

PCR assays were performed to search for *Rickettsia* spp. using the primers described in Table 2 following the protocol described in the original article. DNA samples from *Rickettsia amblyommatis* (from *Amblyomma coelebs*) were used as positives controls; two contamination controls (water) were included as well. PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide (0.5 mg/mL), and visualized in a UV light transilluminator.

Table 2. Sequences of the primers used, together with the respective target genes and the size of the amplified fragment.

Gene/ *Primers*	**Specificity**	**Aim**	**Molecular assay**	**Sequence of *primers* (5'-3')**	**Amplified fragment**	**Reference**
16SrRNA	Family Ixodidae					
16S+		Quality of the extracted DNA	cPCR	CCGGTCTGAACTCAGATCAAGT	460 bp	[24]
16S-				GCTCAATGATTTTTTAAATTGCTGT		
***glt*A**	*Rickettsia* spp.					
CS239 F		Screening	cPCR	GCTCTTCTCATCCTATGGCTATTAT	834 bp	[62]
CS1069 R				CAGGGTCTTCGTGCATTTCTT		
CSF1		Characterization	nPCR	CATCCTATGGCTATTATGCTTGC	885 bp	[63]
CSR1				TATACTCTCTATGWACRTRACC		
CSF2				CTTACCGCTATTAGAATGATTGC	572 bp	
CSR2				GAGCGAKAGCTTCAAGYTCTAT		
17kDa	*Rickettsia* spp.					
17k-5		Characterization	cPCR	GCTTTACAAAATTCTAAAAACCATATA	549 bp	[62]
17k-3				TGTCTATCAATTCACAACTTGCC		
***omp*A**	RSFG					
ompA F1		Characterization	nPCR	GTCAGGCYCTGAAGYTAAACTT	777 bp	[63]
ompA R1				CCATTAACTYAAGCGATTTC		
ompA F2				TGTTGTAGGRACTGCGGYTAAT	460 bp	
ompA R2				ATTAGTAGCAGTAACAACAASTGCT		
Rr190.70p		Characterization	cPCR	ATGGCGAATATTTCTCCAAAA	632 bp	[62]
Rr190-701				GTTCCGTTAATGGCAGCATCT		

RSFG= Rickettsiae Spotted Fever Group; cPCR = convencional Polymerase Chain Reaction; nPCR = Nested Polymerase Chain Raction; bp = base pair

Phylogenetic analysis

The amplified PCR fragments were purified using Exo-Sap-IT (GE Healthcare®), following the manufacturer's protocol, and sequenced in both directions using an automated genetic analyzer (ABI 3730 DNA Analyzer, Thermo Fisher Scientific®) using the Sanger method. The obtained sequences were aligned using the DNA Baser® program and submitted for a homology search with other sequences deposited in GenBank using the BLASTn tool.

Multiple sequence alignments were performed with the sequences obtained in this study and GenBank sequences using MAFFT, using the Jalview v.2.11 software program [25, 26]. The best-fit evolutionary model was determined using MEGA version 11 using the Bayesian information criterion [27]. Phylogenetic relationships were estimated using maximum likelihood (ML) phylogenetic inference with the PhyML tool, using the SeaView v.5 program [28]. The statistical support of the clades was measured using a heuristic search with 1,000 bootstrap repetitions.

RESULTS

Of the 1,885 tick samples tested, 11 were amplified using primers for the *gltA* gene of *Rickettsia* spp*.* There was a prevalence of 0.58% (11/1885) of samples positive for *Rickettsia* spp*.* in relation to the total number of ticks tested in all military areas (Table 3).

Table 3. Tick samples collected from the environment in military training areas in southeastern Brazil, positive for molecular detection of the genus *Rickettsia* spp*.*, by species, stage, MO and area where it was collected.

Ticks				Genes		
Specie	**Stage**	**Nº**	**MO/ Nr Area**	**Positives (%)**	***Rickettsia* spp.**	***RSFG***
A. dubitatum	nymph	3	CIJF/ Area 1	2 (66,67)	2	
A. dubitatum	nymph	1	CIJF/ Area 2	1 (100)	1	
A. dubitatum	nymph	19	CIJF/ Area 3	6 (31,58)	4 2	2
A. sculptum	nymph	120	CIJF/ Area 3	1 (0,08)	1	
A. sculptum	nymph	58	ESA/Area 1	1 (1,72)	1	

Nº= number of tested ticks of the species in the MO area/Nº pos = number of ticks with positive molecular detection by primers targeting the *gltA* and *ompA* genes of *Ricketsia* spp*.* / **MO/Nr Area** =Military instruction area number from where the sample was collected / **CIJF** = Juiz de Fora Instruction Field/Center for Environmental Education and Culture (CIJF/CEAC) / **ESA** = School of Sergeants at Arms / **(%) =** prevalence of positive ticks in the military instruction area relative to the total number of ticks tested in each area*. Rickettsia* spp. = *glt*A and/or *17kDa* gene / RSFG = *Rickettsia*e Spotted Fever Group – *omp*A gene

Of the positive samples, ten came from the CIJF/CEAC and, considering that 290 tick samples were analyzed in this MO, the prevalence of positive samples in this location was 3.45% (10/290). There was also a positive sample from ESA, where 300 ticks were tested, resulting in a prevalence of 0.34% (1/300).

Sequencing of *A. dubitatum* nymphs generated for partial fragments amplified in the *gltA* gene of *Rickettsia* spp*.* was analyzed to determine their identity with sequences from other species deposited in GenBank, showing an identity of 99.56% to 100% with *Rickettsia bellii*. In an *A. sculptum* nymph, 100% identity with *R. bellii* clone Ao#5 (MW384865) was verified, while in another *A. sculptum* nymph, an identity of 98.83% with *Rickettsia* sp*.* ALSK (KX254162), and 98.18% with *R. bellii clone* Ao#5 (MW384865) was verified*.* These findings are referred to in our study as *R. bellii* isolate 313 (GenBank OR365295), *R. bellii* isolate 319 (GenBank OR365296), *R. bellii* isolate 427 (GenBank OR365297), *R. bellii* isolate 446 (GenBank OR365298), *R. bellii* isolate 465 (GenBank OR365299), *R. bellii* isolate 467 (GenBank OR365302), *R. bellii* isolate 468 (GenBank OR365300), and *R. bellii* isolate 469 (GenBank OR365301) for those amplified from the nymphs of *A. dubitatum* and *Rickettsia* sp. isolate 39 (GenBank OR359949) for the *A. sculptum* nymph.

In contrast, two *A. dubitatum* nymphs presented amplified fragments with an identity of 99.82% and 100% with *Rickettsia* sp*.* clone LIC4167A (accession MH158234). When evaluated to amplify partial fragments of the *ompA* gene, the two nymphs of *A. dubitatum* presented 100% (567/567) and 97.8% (577/590) identity with *Rickettsia* endosymbiont of *A. dubitatum* (JN676159) and *Candidatus* Rickettsia colombianensi isolate AdAP (MG970683) (Rr190.70p/Rr190.701 primers) and 97.48% identity with *Rickettsia monacensis* strain IrR/Munich (AH015165) (ompAF1/ompAR1/ompAF2/ompAR2 primers). These samples showed 100% identity with *Rickettsia* sp*.* clone LIC4167A (MH158235) and 99.25% identity with *R. monacensis* strain IrR/Munich (LN794217) when evaluated for the 17 KDa gene. In this study, we referred to these findings as *Rickettsia* sp*.* isolate 463 (GenBank OR359950 - *gltA*, OR365303– *ompA* and OR365306

– *17kDa*) and *Rickettsia* sp. isolate 464 (GenBank OR359951 - *gltA*, OR365304/OR365305– *omp*A and OR365307 – *17kDa*).

Phylogenetic analysis of the amplified genes revealed a close relationship between *R. bellii* and *Rickettsia* sp. Isolate 39 formed a clade with the other *R. bellii* isolates, while isolates 463 and 464 were inserted together with the rickettsiae of the SF group and were phylogenetically very close to *Rickettsia tamurae* and *R. monacensis* (Figures 2, 3 and 4).

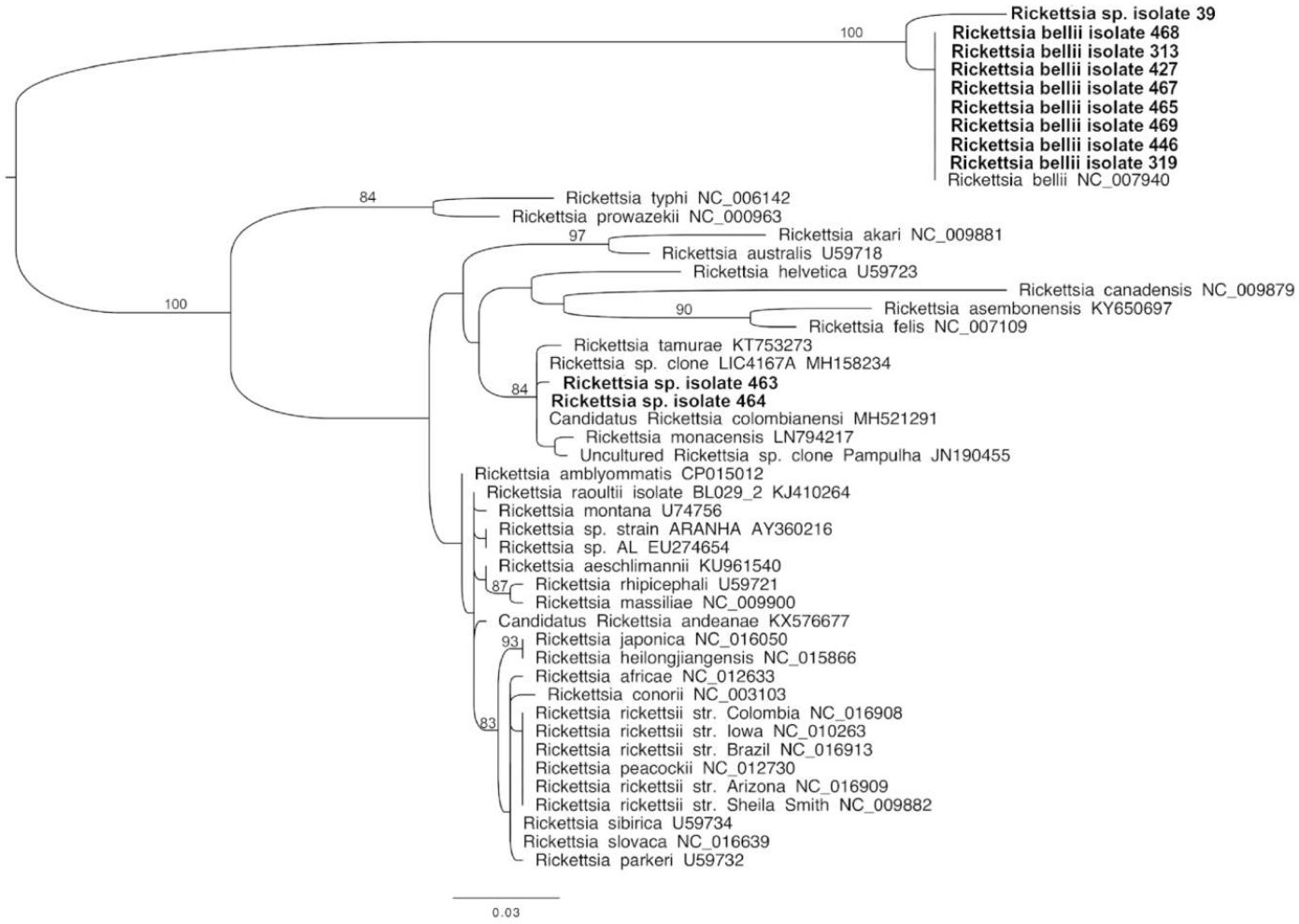

Figure 2. Phylogenetic tree of the *Rickettsia* species identified in this study (in bold), based on the sequences of the *glt*A gene (511nt) of the genus *Rickettsia*, using the Maximum Likelihood method. Numbers (>70%) above branches indicate bootstrap values. Scale bars indicate an evolutionary distance of 0.04 substitutions per sequence position, and branch labels include GenBank accession numbers. The Tamura 3-parameter model with Gamma-distributed heterogeneity (T92+G) was selected as the best-fit evolutionary model.

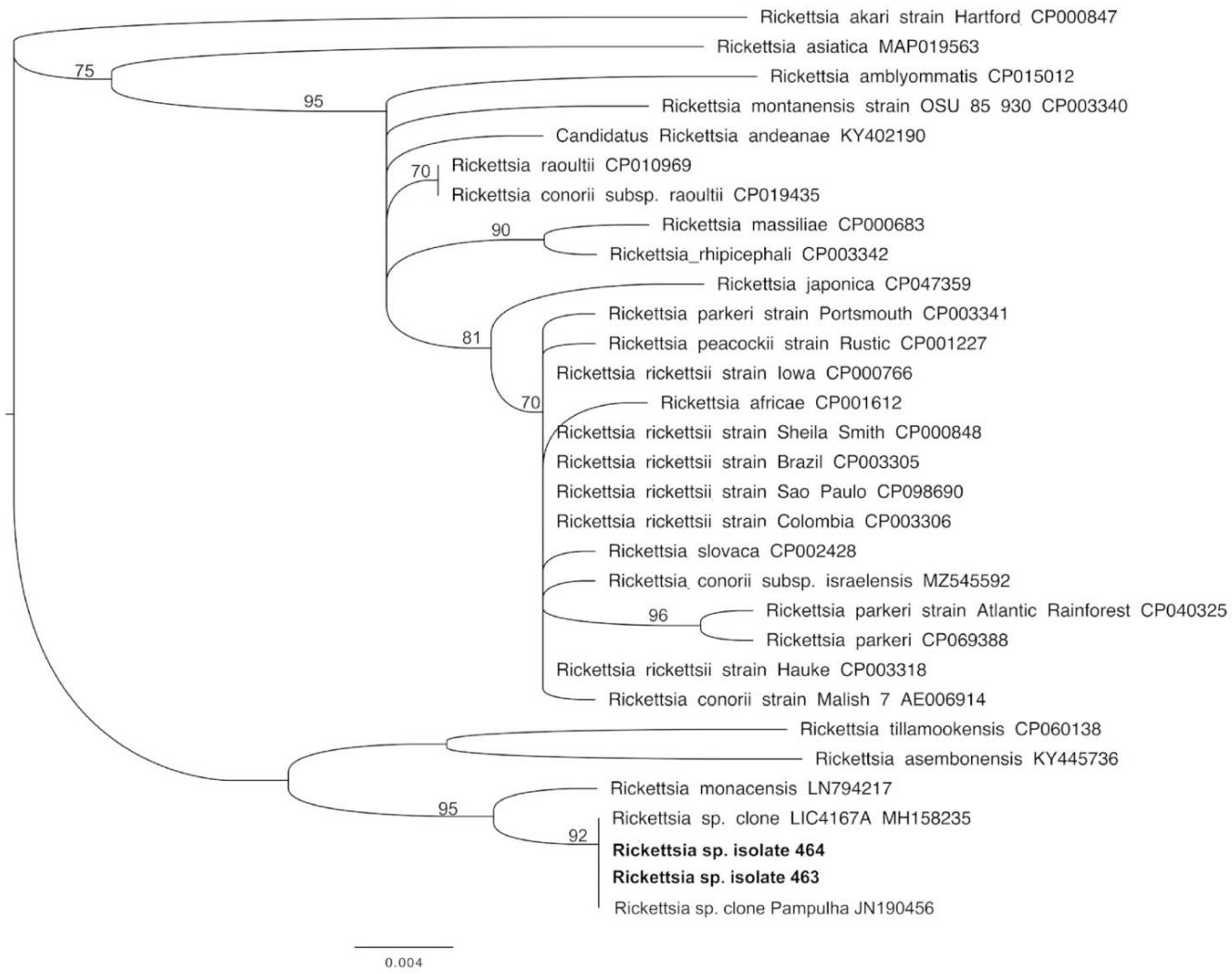

Figure 3. Phylogenetic tree of the *Rickettsia* species identified in this study (in bold), based on the sequences of the 17kDa gene of the genus *Rickettsia* (590 bp), using the Maximum Likelihood method. Numbers (>70%) above branches indicate bootstrap values. Scale bars indicate an evolutionary distance of 0.007 substitutions per sequence position, and branch labels include GenBank accession numbers. The Tamura 3-parameter model assuming that a certain fraction of the sites are evolutionarily invariant (T92+I) was selected as the best-fit evolutionary model.

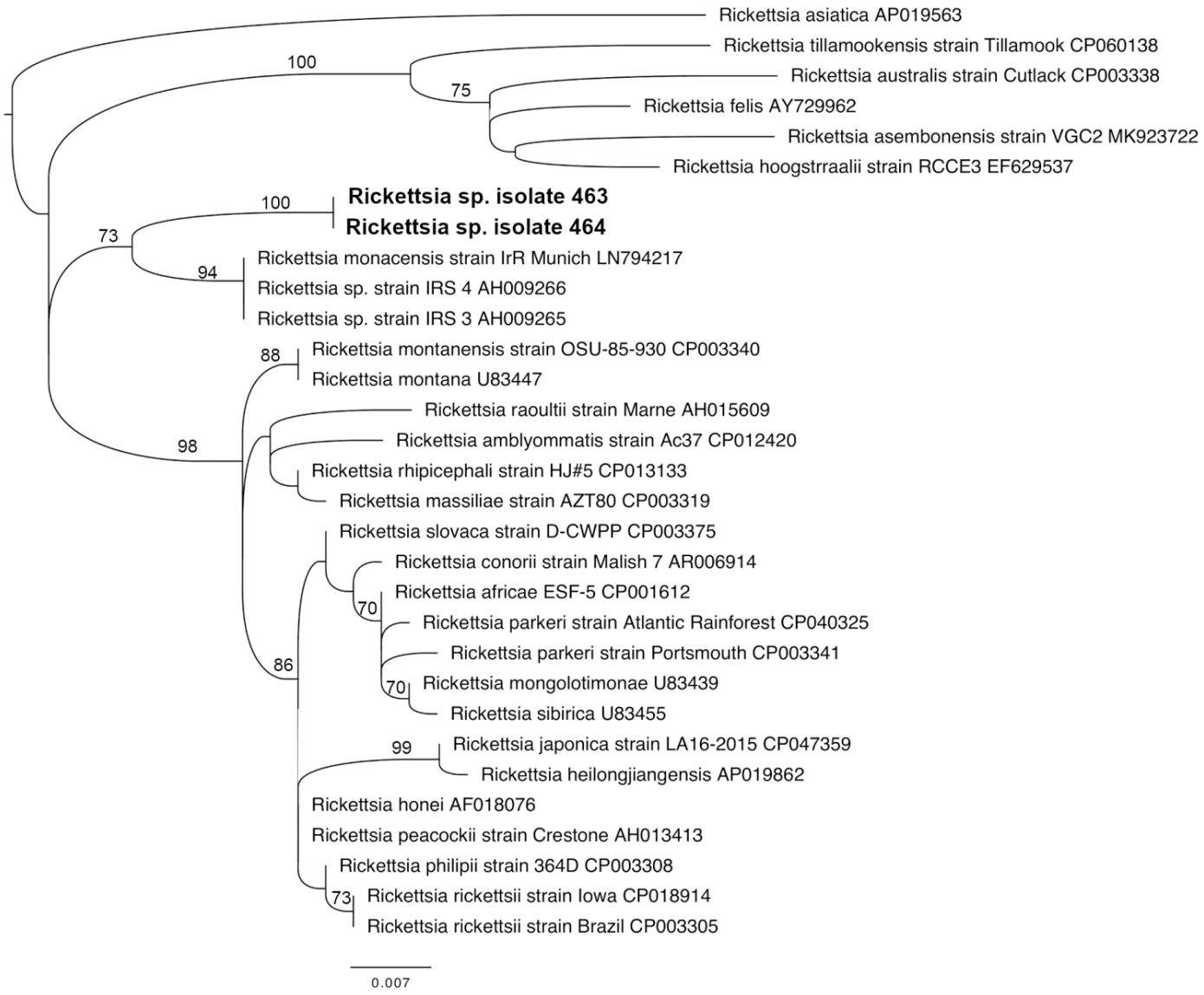

Figure 4. Phylogenetic tree of the *Rickettsia* species identified in this study (in bold), based on the sequences of the ompA gene (Rr190.70p/Rr190.701 primers) of the genus *Rickettsia* (590 bp), using the Maximum Likelihood method. Numbers (>70%) above branches indicate bootstrap values. Scale bars indicate an evolutionary distance of 0.007 substitutions per sequence position, and branch labels include GenBank accession numbers. The Tamura 3-parameter model assuming that a certain fraction of the sites are evolutionarily invariant (T92+I) was selected as the best-fit evolutionary model.

DISCUSSION

This is the first study to analyze the molecular detection of *Rickettsia* spp. in ticks collected from different areas of military instruction frequently used by troops, belonging to different MOs and biomes of the southeast region of Brazil.

Although research analyzing the detection of bioagents infecting ticks in military areas is common in other countries [3,29], little is known about this in military training areas in Brazil. The few surveys carried out in military areas in Brazil recorded the presence of ticks infected with *Rickettsia* spp. in military instruction areas [30], warning of the risk of rickettsiosis among military personnel who make use of these areas.

The results of molecular detection of gene sequences with identity for *Rickettsia* spp. in *A. sculptum* and *A. dubitatum* ticks collected in areas of the CIJF/CEAC, in Juiz de Fora, MG, and the ESA, in Três Corações, MG, corroborate the previously mentioned findings, reinforcing the need for epidemiological and acarological surveillance in military training areas [30].

Minas Gerais has shown a high number of Brazilian SF cases and deaths, and the microregion of Juiz de Fora stands out in the state scenario [31, 32]. This municipality has vulnerability indicators for BSF [31], with outbreaks of the disease [33, 34] and frequent records of confirmed cases in the municipality in its micro-

region [35]. There are no similar surveys previously conducted in the municipality of Três Corações, but there are recent records of two confirmed cases of BSF in the municipal area [35].

Research carried out in Juiz de Fora, a region considered endemic for BSF, has provided molecular evidence of the presence of rickettsiae in ticks in the microregion, such as *R. bellii* in *A. dubitatum,* and *R. rickettsii* and *R. amblyommatis* in *A. sculptum* [31, 36]. Thus, the results of the present study are in line with previous research carried out in the region, corroborating the presence of rickettsiae with identity with *R. bellii*, in nymphs of *A. dubitatum* and *A. sculptum*, and *Rickettsia* sp. clone LIC4167A in nymphs of *A. dubitatum* from areas of military instruction in the municipality of Juiz de Fora, MG.

Despite serological evidence of transmission of *R. bellii* from tick vectors to vertebrate hosts [37], its pathogenicity has not been fully elucidated in humans and, to the best of our knowledge, there are no recorded cases of SF related to this rickettsiae [9,38].

In contrast, two isolates of *Rickettsia* sp*.* detected in *A. dubitatum* nymphs have been reported to have close identity and close phylogenetic relationships to pathogenic rickettsiae of the SF Group, such as *R. monacensis* and *R. tamurae* [39]. These isolates, referred to herein as *Rickettsia* sp*.* isolate 463 and *Rickettsia* sp*.* isolate 464, have 99.9% and 100% identity, respectively, with the *Rickettsia* endosymbiont of *A. dubitatum* (JN676158), *Rickettsia* endosymbiont of *Ixodes tapirus* (MW699691), *Rickettsia* sp*.* clone LIC4167A (MH158234), and Candidatus *Rickettsia colombianensi* (MW384861) described in *A. dubitatum* [40], *I. tapirus* [41], *A. sculptum* [39], and *Amblyomma dissimile* [38]. According to the criteria proposed by Fournier and coauthors [42], when a species has an identity above 99.9% for the *gltA* gene, it refers to the same species. Because only a fragment of the gene is being analyzed, we would possibly be facing a species of the genus *Rickettsia* with multiple vector hosts. However, analyzing the fragment of the *omp*A gene, none of these isolates has an identity greater than 98.8% (criterion proposed by Fournier and coauthors [42]), except for *Rickettsia* endosymbiont of *A*. *dubitatum* (JN676158) with identity 100% (567/567).

Although little is known about the pathogenic potential in humans, there are records of human cases of SF caused by *R. monacensis* on the European continent [43-45] and *R. tamurae* in Japan [46], which are considered pathogenic species for humans [9]. *Amblyomma dubitatum* is considered to be less aggressive to humans, and human infestation by this species is uncommon [47-49].

However, the 100% similarity with the finding by Sato and coauthors [39] described in a male *A. sculptum* ticks, collected from a dog, in the region of Pedro Leopoldo, MG, during an epidemiological survey in an area endemic of SF, alerts to the need for further studies on this rickettsia, given its possible parasitic potential for humans, with the possibility of involvement in the different forms of SF presentation and eco-epidemiological cycles that have been discovered in our country [39, 50].

Finally, it should be noted that the absence of molecular detection of evidence of *Rickettsia* spp*.* in ticks in other surveyed areas in no way rules out the possibility of this fact occurring nor certifies such areas as free of rickettsiae. Because this is a cross-sectional study, there is a major limitation in the detection of the circulation of this bioagent [51]. Furthermore, the prevalence of *R. rickettsii* infection of ticks in the free-living phase is very low because of its deleterious effect on vectors, which also makes detection difficult [52]. Although the present study did not test ticks of the species *Amblyomma ovale*, the opposite was observed in relation to infection by *R. parkeri* strain “Mata Atlântica” in ticks of this species collected from the environment, with a prevalence ranging from 5% to 85% [53, 54].

As verified by Prado and coauthors [22], the surveyed areas gather vulnerability factors for the occurrence of BSF cases, being classified as “predisposed areas” to the occurrence of human cases [55, 56]. Recent lethal cases of BSF caused by *R. rickettsii* occurred in military police officers after training in a forested area in Campo Grande, RJ, and in a soldier after field activity in a military training area in Campinas, SP, reinforcing this predisposition and occupational risk of these professionals for the disease [57-59].

Molecular evidence for the presence of *Rickettsia* spp*.* in *A. sculptum* and *A. dubitatum* ticks from military training areas indicates the risk of occupational exposure of military personnel to ticks infected with *Rickettsia* spp*.* during their training, predisposing these professionals to rickettsiosis, as has been highlighted by research conducted in Brazil [30, 60] and abroad [18, 29].

It is necessary to carry out more research, such as serology in sentinel animals in addition to continuous monitoring and acarological control in these MOs. The prevention and mitigation of occupational biological risks is a strategic factor for the protection of force health [3, 29, 61]. Through these surveys, it will be possible to understand and better measure such risks and define the classification of areas, collaborating for adequate management of the health risks existing in each location.

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