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Original Article

Molecular detection of *Rickettsia africae* in *Amblyomma* ticks collected in cattle from Southern and Central Mozambique

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Abstract

Introduction*: Rickettsia* are Gram-negative and obligate intracellular bacteria, which cause typhus and spotted fever-like diseases in humans. In Africa, *Rickettsia africae* of the Spotted Fever Group *Rickettsia* (SFGR) is the etiologic agent of the African Tick-Bite Fever. The disease is transmitted by ticks of the genus *Amblyomma*, which serve as vectors and reservoirs of *Rickettsia*. In this study, we aimed to detect *Rickettsia* species in ticks collected from cattle in south and central Mozambique.

Methodology: DNA from 412 adult ticks and 22 pools of larvae were extracted and tested for the presence of *Rickettsia* genes *gltA, ompA* and *ompB* by PCR, followed by sequencing and phylogenetic analysis.

Results: Our results showed that in adult ticks, 79.5% (n = 330), 66% (n = 274) and 67% (n = 275) samples were positive for *gltA*, *ompA* and *ompB* genes, respectively. Among the 22 pools of larvae analysed, 77.2% (n = 17) were positive for the three genes tested. The infection rates ranged from 43% to 100% for *Rickettsia* by *gltA* in all locations studied, with maximum values of 100% observed in the districts of Maputo province namely Changalane, Boane and Matutuine district. The phylogenetic analysis of amplified sequences revealed that samples under study grouped with *R. africae* for the 3 genes.

Conclusion: The study showed that Spotted Fever Group *Rickettsia* represented by *R. africae* widely circulate in *Amblyomma* ticks collected in south and central regions of Mozambique.

Key words: Spotted fever group Rickettsia*; Amblyomma*; *R. africae*; ATBF.

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Introduction

The genus *Rickettsia* comprises obligate intracellular bacteria [1], among which 22 species have been proven to be pathogenic to humans [2,3]. This genus is divided into three taxonomic groups defined according to the distinctive diseases they cause, namely, the typhus group (TG), spotted fever group *Rickettsia* (SFGR) and scrub-typhus groups (STG), each comprising several *Rickettsia* species [3]. The three groups differ in terms of the clinical symptoms they cause, on the basis of host specificity, [4,5], *in vitro* growth conditions, antigenic characteristics, the molecular sequences of conserved genes [3,6,7], clinical features, guanine and cytosine (G + C) content of the genome and intracellular localization [5,8].

SFG rickettsiae include *Rickettsia conorii*, *Rickettsia africae*, *Rickettsia aeschlimannii*, *Rickettsia sibirica* and *Rickettsia mongolotimonae* [9] transmitted by ixodid ticks [10]. The TG consists of *R. prowazekii* and *R. typhi,* which is transmitted by fleas and lice [11]. STG rickettsiae comprise the mite-transmitted *Orientia tsutsugamushi* previously called *R. tsutsugamushi* [12], of which chiggers are the main vectors [13].

Within the SFGR, the species *R. africae* is the causative agent of the African Tick-Bite Fever (ATBF), a tick-borne disease which was long mistaken for the Mediterranean spotted fever (MSF) caused by *R. conorii* [14]. Over 100 years ago, MC Naught and Sant'Anna reported what is likely to have been the first description of human cases of ATBF in Mozambique and South Africa [14]. This initially led to a debate as to whether these cases were indeed the result of a new type of infection, and not MSF, a disease that had been described in Tunis in 1910 [15]. ATBF causes moderately severe clinical features, including fever, headache, myalgia and often an eschar at the site of the tick bite, but can become more serious for elderly and immunocompromised people [16,17].

Recently, the implementation of molecular methods for diagnostics has aided the discovery of several new

species of *Rickettsiae* worldwide [18]. However, despite the increasing awareness of the importance of emerging rickettsial diseases, there is still a lack of information on the prevalence of SFGR *Rickettsiae*, especially in countries such as Mozambique, where diagnostic capacity is limited.

Tick-borne spotted fever is the most frequently reported type of travel-associated rickettsial infection, and the African tick-bite fever is the most commonly reported in Africa [14,19,20]. However, even though *R. africae* is widely distributed in sub-Saharan countries [21], and sero-surveys have shown that infections are extremely common in humans [22,23], reports on ATBF in indigenous people are unexpectedly rare [24]. This could be due to the difficulty of proper diagnosis for infections caused by *Rickettsia*, especially when people are infected at a young age and medical attention is less likely to be sought [24]. Furthermore, the lack of sensitive and specific tests contribute to hinder the proper diagnostic capacity in rural areas where the infections are more likely to occur [25].

In Southern Africa, ticks of the species *Amblyomma hebraeum* are recognized as vectors and reservoirs of *R. africae,* which has been demonstrated to be transovarially and transtadially transmitted in *Amblyomma* ticks [24]. ATBF caused by *R. africae* and transmitted by *Amblyomma* ticks has been reported in neighboring countries such as Zimbabwe and South Africa [14,19,26,27,28]. However, its presence in ticks from Mozambique has not yet been confirmed. Additionally, the extent of zoonotic transmission and the contribution of this agent in cases of non-malarial acute febrile illness in humans in Mozambique is currently unknown, emphasizing the importance of conducting epidemiological studies involving hosts and vectors.

Therefore, this study was designed with the aim of assessing the infection rate of *Rickettsia spp,* and especially *R. africae,* in ticks collected from autochthonous cattle from southern and central regions of Mozambique, using a molecular approach.

Methodology

Tick collection

From March to August 2013, adult ticks and larvae of *Amblyomma spp.* were collected from cattle. Adult ticks were collected in 12 districts of the southern and central regions of Mozambique, in Maputo, Inhambane, Sofala and Manica Province.

For convenience, the cattle from which the adult ticks were collected (n = 30) were selected based on the criterion of good tick visibility on the animals. Ticks were removed manually and stored into tubes containing 70% ethanol. Each tube was labeled according to the place of collection (district), species, sex and collection date.

Larvae were collected from pastures in the South of the country by dragging, pooled into tubes with 10-20 larvae and kept in 70% ethanol. Subsequently, adult ticks and larvae were submitted to taxonomic identification at the Biotechnology Center of Eduardo Mondlane University (CB-UEM) laboratory, based on the entomological keys of Walker *et al*. [29].

DNA extraction

Adult ticks were weighed, and those within the 25-40 mg range were included in the study. Ticks were subsequently washed five times using phosphate buffer saline (PBS) and cut in small pieces to be processed for extraction. Tick and larvae DNA extraction was performed using the QIAamp DNA Blood Mini kit 250 Cat. No 51106 (Qiagen, Hilden, Germany), following the manufacturer's instructions. The DNA was stored at -20° C for further molecular analysis. Samples were identified according to each locality with a three-digit code referring to the three initial letters of the collection location as follows: Cha for Changalane; Manh for Manhiça; Mat for Matutuíne; Boa for Boane; Mab for Mabote; Mbn for Mambone; Mass for Massinga; Msr for Mossurize; Mgd for Magude; Mch for Machaze; Ins for Inhassoro and Chi for Chibabava.

PCR detection

Screening of Rickettsial DNA was performed using *Rickettsia* specific PCR assay for *gltA, ompA* and *ompB* genes, chosen according to previously published studies on *Rickettsia* detection [2]. Two primer sets, CS2-F/R and CS239/CS1069 for the citrate synthase gene (*gltA*) present in all *Rickettsia spp* (SFGR and TG), were used to amplify fragments of 401 bp and 830 bp, respectively [2].

The reaction mixture consisted of 2 µL of extracted gDNA, 5 µL of PCR master mix (5x Green Go Taq Buffer, Promega, Madison, USA), 1.25 µL each of forward and reverse primers, 14.75 µL of sterile water and 0.25 µL of Go Taq. Negative and positive controls for the PCR were sterile water and *R. conorii* DNA from cell culture. The reaction was carried out in a PCR Applied BioSystem machine (Gene Amp® PCR System 9700, Foster City, California, USA) with the following conditions: initial denaturation at 95°C for three minutes, followed by 40 cycles of 95°C for 15 seconds, 48°C for 30 seconds, 72°C for 30 seconds,

with an additional extension period of 72°C for seven minutes on the final cycle.

Furthermore, samples positive to *gltA*, were submitted to PCR using primers Rr190k.720n/Rr190k.71p and RICI/RICII specific for *ompA* and *ompB* genes, which amplify fragments of 650 bp and 500 bp, respectively [6,30]. The PCR was conducted according to the conditions validated in previous studies [2]. After PCR, 5 µL of the products were electrophoresed on 1.5% of agarose gel stained with Green DNA stain (Thermo Fisher Scientific, Waltman, MA, USA), DNA bands were visualized on a trans-illuminator model TFX-20-M (6 × 15 W).

Limit of detection

To analyze the limit of detection and robustness of the primers, primers CS2-F/CS2-R Rr190k71p/Rr190k70n and RICI/RICII were tested in triplicate with a range of 10 ng to 1 fg (1 fg = 1×10^{-6} ng) of purified genomic DNA, (1 genomic copy assuming the size of *Rickettsia* genome as of ~1Mbp = 1fg). *Rickettsia* DNA with known concentration (18.6 ng/µL) was serially diluted to obtain quantities ranging from ~10 ng to ~1 fg. Dilutions were visualized on agarose gel for quality and integrity control.

Furthermore, a sample containing only tick DNA was included in the reaction to test the potential inhibitory effect of tick DNA on *Rickettsia* detection. After mixing, total DNA (tick DNA and *Rickettsia* DNA) was diluted 10X and followed the same procedure as the positive control in the previous step. Finally, each pair of primers was tested using 3 positive samples for *Rickettsia*, namely Cha2, Cha30 and Cha29 with known concentrations of 24.9 ng/µL, 131.1 ng/µL and 162.1 ng/µL, respectively. The PCR was conducted in 3 replicates following the procedure mentioned above in a 1:10 serial dilution. The PCR product was visualized on 1.5% agarose gel for DNA integrity control and to assess the limit of detection.

Sequencing and phylogenetic analysis

Selected PCR products of the genes *gltA*, *ompA* and *ompB* were purified using the NEB cleanup kit (New England Biolabs, Ipswich, Massachusetts, USA). Five microliter of purified PCR product were mixed to 10 pmol of the appropriate primer and sent to Macrogen Europe for sequencing (https://dna.macrogen-europe.com/eng/). The sequences obtained were used to determine the phylogeny through a comparative analysis with sequences of *Rickettsia sp* available in Genbank retrieved by BLASTn [31]. Sequences were aligned using Clustal X version 2.0 (Conway Institute UCD Dublin, Ireland) [32]. Phylogenetic trees were produced using Bayesian Inference (BY). BY phylogenetic reconstructions were performed using the HKY85 substitution model by running 1×10^{6} generations, with Markov chains sampled every 1000 generations. A burn-in of 10% was applied and the remaining trees were used to compute a 50% majority rule consensus tree and posterior probabilities.

Results

Tick collection

Out of a total of 387 *Amblyomma* ticks, 357 *A. hebraeum* adult ticks were collected in eleven (11) districts from four (4) provinces, namely Maputo (n = 176), Inhambane (n = 97), Manica (n = 82) and Sofala (n = 2) provinces; *A. variegatum* adult ticks (n = 30), were just collected in Govuro district, Inhambane province.

Larvae were only collected in Matutuíne and Changalane districts, and in the Veterinary Faculty campus, Eduardo Mondlane University. Specifically, 22 pools of larvae were collected in the study: 12 from the Veterinary Faculty campus, 7 from Changalane and 3 from Matutuíne districts in Maputo province (Figure 1).

Rickettsiae infection rates

Of the 357 *A. hebraeum* adults, 80% (n = 287) were positive for *Rickettsia* (% of *gltA* positive samples/total samples), and 65% (n = 231) were positive for the *ompA* and *ompB* genes. Of the 30 *A. variegatum* adult ticks, 67% (n = 20) were infected by *Rickettsia*, and 73% (n = 22) and 77% (n = 23) were positive for the *ompA* and *ompB* genes, respectively.

When looking at the *Rickettsia* infection rates in adult tick per district, *A. hebraeum* ticks ranged from 43% to 100%, with the highest values (100%) detected in ticks collected in districts of the Maputo province, namely Changalane, Boane and Matutuine. The lower infection rate (43%) was observed in ticks collected in Inhambane Province. Adult *A. variegatum* ticks collected in Inhambane showed an infection rate of 67%.

Larvae collected in the districts of Maputo Province showed *Rickettsia* infection rates ranging between 71 % and 75% in Changalane district and the Veterinary Faculty campus, while a 100% of infection rate was observed in Matutuíne district (Figure 1).

Figure 1. Map of the study area, showing each district sampled. Adults *Amblyomma* ticks captured per district. B: *Amblyomma* larvae captured.

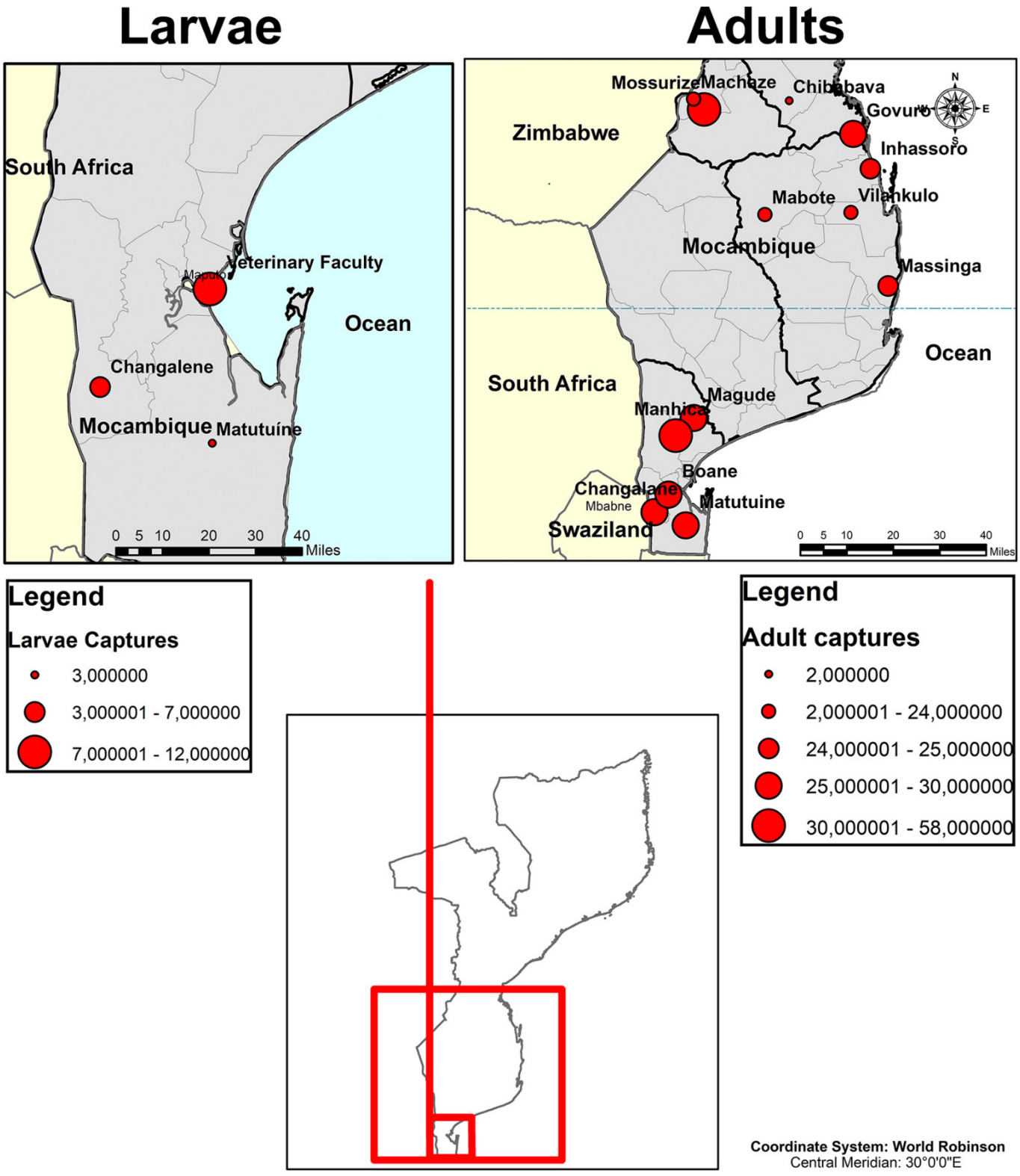


Table 1. Limit of detection for each dilution per primer and gene tested in the study.

DNA concetration	Dilution	Genomic copies	CS2F/CS2R	Rr190k71p/ Rr190k.720n	RICI/ RICII
18.6 ng/µL	10^{0}	1×10^{7}	+	+	+
1.86 ng/µL	10^{-1}	1×10^{6}	+	+	+
186 pg/µL	10^{-2}	1×10^{5}	+	+	+
18.6 pg/µL	10^{-3}	1×10^{4}	-	-	-
1.86 pg/µL	10^{-4}	1×10^{3}	-	-	-
186 fg/µL	10^{-5}	1×10^{2}	-	-	-
18.6 fg/µL	10^{-6}	10	-	-	-
1.86 fg/µL	10^{-7}	1	-	-	-

Limit of detection

All three primer pairs tested (CS2F/CS2R, Rr190k71p/Rr190k.720n, and RICI/RICII) had a low limit of detection of 18.6×10^{-2} ng/µL for *Rickettsia* DNA, which is equivalent to 10^{5} genomic copies.

The limit of detection of *Rickettsia* DNA for the three primers, remained the same in the presence of tick DNA (10^{-2} equivalent to 10^{5} copies of genomic DNA) (Table 1).

Confirmation of Rickettsiae species by sequencing

A total of 32 samples were sequenced for the *gltA, ompA* and *ompB* genes. After trimming and BLASTn comparison with publicly available *Rickettsia* sequences, low quality sequences (short, weak signal) were eliminated, resulting in 10, 10 and 7 good quality sequences for *gltA, ompB* and o*mpA,* respectively.

All 10 *gltA* sequences showed values of > 98% sequence similarity to *R. africae* (Accession number KX227776). Similar results were obtained for the *ompA* and *ompB* genes, where the *ompA* sequence homology ranged from 99% to 100% to *R. africae strain ESF* (Accession number U83436) and *ompB* sequence homology ranged between 97% and 99% for *R. africae* (Accession no KF660533) (Table 2).

Unexpectedly, one sample isolated from *A. variegatum* matched with *Ehrlichia ruminantium* (Accession number CR925677) with 87% of sequence similarity.

Phylogenetic analyses

The phylogenetic analysis generated by separate alignments of the three amplified genes confirmed that *Rickettsia* detected in this study belonged to the *R. africae* species (Figure 2 and Figure 3). The phylogenetic tree originated by the alignment of 10 *gltA* gene amplicons and 23 reference sequences showed that all the samples from this study clustered within the *R. africae* group (Figure 2). These results were also confirmed by the alignment of the *ompA* gene with reference strains (data not shown).

Table 2. Percentage of identity of amplified genes to reference strains of *R. africae*. Strains under study are designed by: Cha- Changalane; Manh- Manhiça; Mass- Massinga; Msr- Mussorise; Mbn-Mambone. Dash symbols indicate that the gene was not sequenced for that specific strain.

Sample	***gltA* (Acc. no. KX227776)**	***ompB* (Acc. no. KF660533)**	***ompA* (Acc. no. U83436)**
Cha_3	99%	99%	99%
Cha_9	99%	-	99%
Cha_20	99%	-	99%
Manh_1	99%	99%	99%
Manh_16	-	99%	-
Mass_2	99%	99%	99%
Mass_7	-	97%	-
Mass_15	99%	-	99%
Msr_168	99%	99%	99%
Mbn_1	99%	-	-
Mbn_2	98%	-	-
Mbn_7	-	99%	-
Mbn_14	-	98%	-
Mbn_21	-	-	-
Mbn_23	-	96%	-
Mbn_26	98%	-	-
Mbn_29	-	98%	-

Figure 2. Bayesian based phylogenetic tree on partial *gltA* sequences showing the phylogenetic placement of *R. africae* strains among *Rickettsia* species. Numbers at nodes indicate values of posterior probabilities. Strains from this study are indicated in orange.

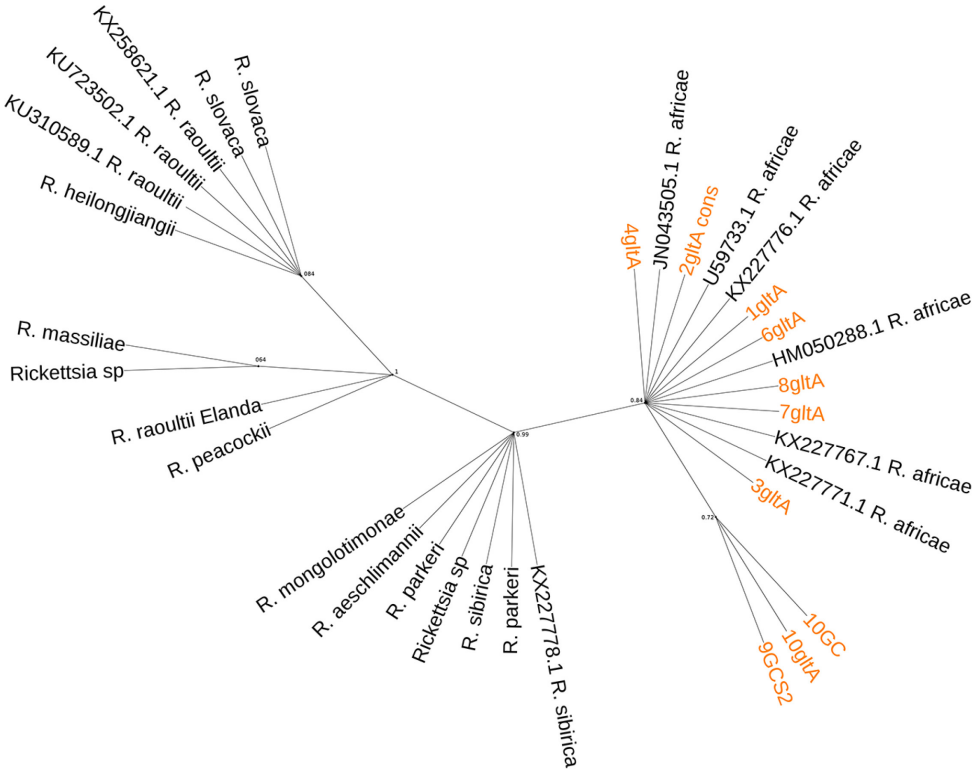
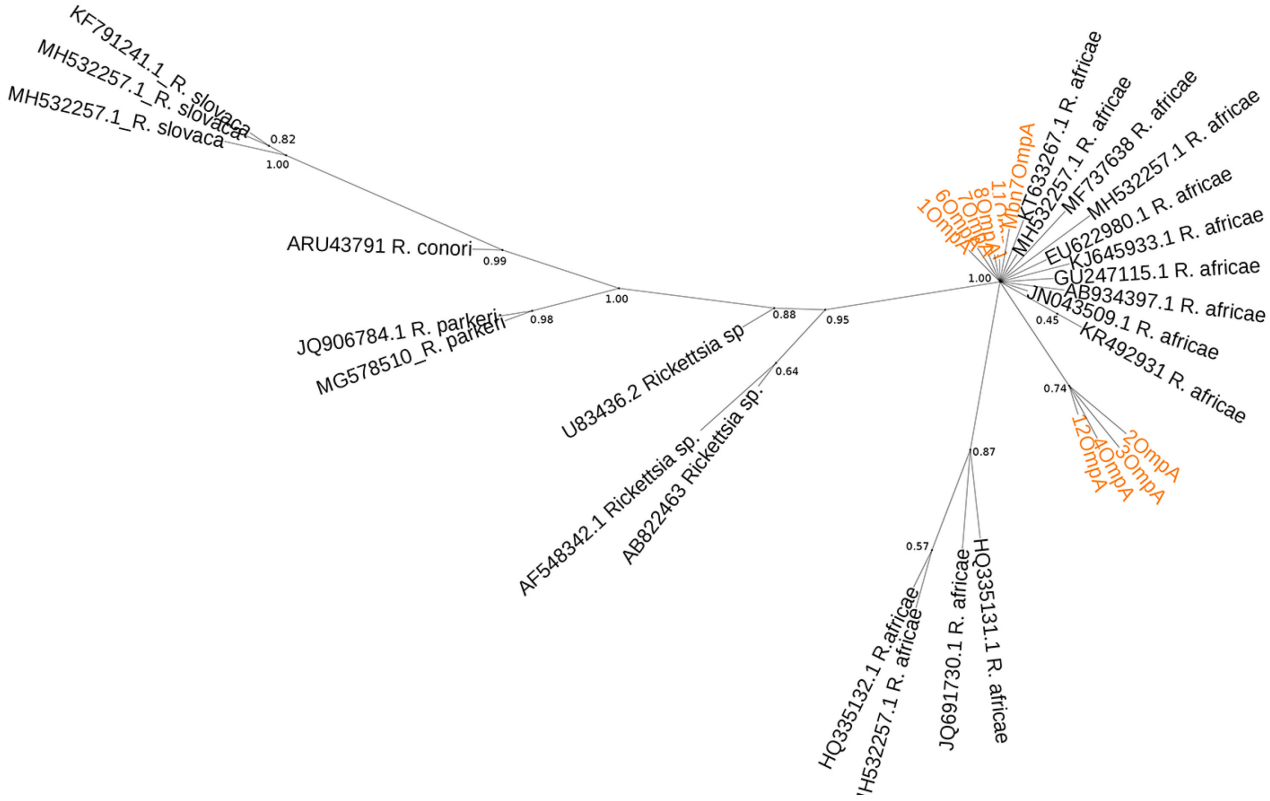


Figure 3. Bayesian based phylogenetic tree on partial *ompA* sequences showing the phylogenetic placement of *R. africae* strains among *Rickettsia* species. Numbers at nodes indicate values of posterior probabilities. Strains from this study are indicated in orange.



Furthermore, an *in silico* restriction map (RFLP *in silico*) for the *ompA* gene was made using the sample under study and reference sequences (Figure not shown). The restriction analysis demonstrated that our samples had the same restriction profile as the reference sequences. This indicates that we are working with the same group of samples, which confirms the clustering observed in the phylogenetic trees.

Discussion

In this study, *R. africae* DNA was detected in *A. variegatum* and *A. heabreum* ticks collected from southern and central regions of Mozambique. Previous studies reported that *A. hebraeum* and *A. variegatum* were vectors and reservoirs of *R. africae* in some regions of Africa where there is a close interaction between humans and cattle parasitized by ticks [14, 25, 33,34]. The results of this study confirm, for the first time, the circulation of *R. africae,* the causative agent of ATBF in *A. hebraeum* and *A. variegatum,* in Mozambique.

Southern African countries such as Zimbabwe, South Africa, Lesotho, and Swaziland, have also reported infection in ticks and transmission of *R. africae* to humans [22,23]. Specifically, in Zimbabwe*, R. africae* infection rates above 80% were reported in *A. hebraeum* [35,36]. Similarly, in our study 80% of adult *A. hebraeum* ticks were infected with *R. africae*. The high infection rate observed is probably due to transovarial transmission in ticks [34,36], which is consistently supported by an infection rate of 77%, found in pools of non-feeding *A. hebraeum* larvae recovered from pastures in Maputo Province. Both these results confirm the role of *A. hebraeum* ticks as reservoirs of *R. africae* in Mozambique.

Moreover, even though *A. hebraeum* is the main vector of *R. africae* in southern Africa, *A. variegatum* also plays an important role as a vector and is widely distributed in sub-Saharan Africa [24,37-39]. Our results show that *A. variegatum* is also a potential vector of *R. africae* in Mozambique, with an infection rate of 73%. In Mozambique, *A. variegatum* is present in all north and central regions of the country [40], while *A. hebraeum* is widespread in all the provinces south of Save River [41].

Although our results confirm the presence of the pathogen and the vector role of *Amblyomma* species using molecular approaches, these do not allow for a complete assessment of vector capacity. However, studies conducted by Socolovschi *et al*, [35] and Kelly & Manson [42] have experimentally proven the transmission of *R. conorii* and *R. africae* by *A. hebraeum* and *A. variegatum* and its role as a vector of *Rickettsia*.

The high rate of *R. africae* infection in ticks of the species *A. hebraeum* and *A. variegatum* reported in the present study suggests that the transmission to humans by these species should be considered a significant public health issue in the southern and central regions of Mozambique.

The presence of *Rickettsia* species of the SFG in ticks, and the significant degree of habitat sharing between domestic and wild animal hosts increase the likelihood of the pathogen's circulation in this habitat. The abundance of infected ticks at the sites included in this study, combined with the proximity between humans and domestic animals, suggest a high probability of infections occurring within rural human populations of Mozambique, where the problem is generally underestimated due to a lack of proper diagnosis. This scenario points to a possible occurrence of silent outbreaks of the disease, or to the existence of an endemic stability within the rural communities.

Conclusions

Through the use of molecular detection methods, this study confirmed the presence of SFGR *R. africae* in *A. hebraeum* and *A. variegatum* ticks, circulating in the south and central regions of Mozambique. Their geographic distribution in the studied areas and observed infection rates point to a potential underestimated threat to human health. It is, therefore, necessary to conduct further epidemiological studies involving hosts and vectors in domestic animals and human population interfaces to characterize the transmission of *Rickettsia* between these groups.

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Author contributions

VM and NC participated in tick collections, tick idetification, conducted DNA extraction. VM performed *R. africae* PCR screening, data analysis and wrote the article. ET and LN,

designed and supervised the study. All authors edited and approved the final manuscript.

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