*Am. J. Trop. Med. Hyg.*, 50(3), 1994, pp. 373–380
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ISSN: 0002-9637

# IDENTIFICATION OF RICKETTSIAE FROM TICKS COLLECTED IN THE CENTRAL AFRICAN REPUBLIC USING THE POLYMERASE CHAIN REACTION

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*Abstract.* Spotted fever rickettsiosis have been identified on the African continent since their historical description in 1909. However, only *Rickettsia conorii* and *R. africae* have been described in Africa, and the current techniques for the detection of rickettsiae in ticks are difficult to apply in large field studies. We report here a preliminary study using genomic amplification by the polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) analysis directly on 310 crushed ticks (*Rhipicephalus, Amblyomma,* and *Haemaphysalis* species) collected in 1985 in the Central African Republic. Among 310 specimen tested, 21.6% were positive. The rate of infection ranged from 0% to 64.3%, depending on the tick species. Based on PCR-RFLP, five different rickettsiae profiles were found: *R. conorii* and *R. africae,* previously known in Africa, *R. rhipicephali,* which has never been described in Africa, and two isolates identical to *R. massiliae* and Mtu5, previously obtained from *Rh. turanicus* in southern France. This work shows that PCR-RFLP is a powerful tool to study tick collections, and that it is applicable to samples from developing countries. Further work is needed to confirm the identification of the rickettsiae found in this work, using traditional identification procedures.

Mediterranean spotted fever, a tick-borne rickettsial disease of humans, was first described by Conor and Bruch in Tunisia in 1910.[1] Since then, similar diseases, first related to *Rickettsia conorii,* have been described in Africa as tick bite fever, South African spotted fever, or Kenya tick typhus.

The epidemiology of rickettsiae and rickettsial diseases in Africa is still poorly known. A few recent seroepidemiologic studies have shown their presence throughout the continent.[2–5] Although these studies provided important information about the prevalence of known pathogenic rickettsiae, they should be expanded as part of larger epidemiologic studies that include the identification and ideally, the isolation of the rickettsiae. Large-scale tick studies are difficult to carry out because classic techniques are time- and material-consuming and they require viable ticks for hemolymph testing or freezing at −80°C for shell-vial centrifugation.[6–8]

Recently, polymerase chain reaction (PCR) technology has been applied to the detection of rickettsial DNA in isolates[9] and in various other specimens.[10–14] We have applied this technique to a collection of triturated ticks from the Central African Republic that have been frozen for six years.

## MATERIALS AND METHODS

### *Tick collection and processing*

Three hundred ten ticks were collected in 1985 in the abattoir of Bangui, Central African Republic. They were collected from cattle (species N'Dama and Baoule) from the areas of Bouar (5°58′N, 15°38′E), Bangassou (4°45′N, 22°50′E), and Bambari (5°46′N, 20°40′E). After identification by an entomologist (J-PC), they were triturated in culture medium (Hanks' medium containing 0.75% bovine albumin and antibiotics) and frozen at −80°C. The samples have been subsequently thawed and frozen at least three times for previous studies and sorting. The species distribution was as follows: *Amblyomma variegatum:* 22, *Rhipicephalus senegalensis:* 28, *Rh. mushamae:* 144, *Rh. lunulatus:* 46, *Rh. sulcatus:* 32, *Rh. gr. compositus:* 14, *Haemaphysalis paraleachi:* 21, *H. punctaleachi:* 2, and *H. gr. leachi:* 1.

Each sample was thawed, resuspended, and separated in two aliquots. One was used for the present work, and the other one was frozen at −80°C for further studies. The aliquots used in this study were washed in sterile distilled water five times by centrifugation (3,500 × *g* for 5 min), resuspended in 100 μl of sterile distilled



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water, and heated at 100°C for 10 min. Ten microliters of the heated suspension was used in the PCR mixture.

*Polymerase chain reaction*

**Oligonucleotide primers.** Two sets of primers were systematically used for all samples. The first, *Rp* CS.877p (5′-GGG-GGC-CTG-CTC-AGC-GCG-C-3′) and *Rp* CS.1258n (5′-ATT-GCA-AAA-AGT-ACA-GTG-AAC-A-3′), primed the citrate synthase gene of typhus- and spotted fever group (SFG)–rickettsiae and produced a 381-basepair (bp) fragment.[9, 15] The second, *Rr* 190.70p (5′-ATG-GCG-AAT-ATT-TCT-CCA-AAA-3′) and *Rr* 190.602n (5′-AGT-GCA-GCA-TTC-GCT-CCC-CCT-3′) primed the 190-kD antigen gene of SFG rickettsiae only and produced a 532-bp fragment.[9, 16]

A third set of primers, BG 1-21 (5′-GGC-AAT-TAA-TAT-CGC-TGA-CGG-3′) and BG 2-20 (5′-GCA-TCT-GCA-CTA-GCA-CTT-TC-3′), which primed the 120-kD antigen gene of SFG rickettsiae only and produced a 650-bp fragment, was used for the samples in which the pattern of the Israelian tick typhus (Itt) rickettsia was detected to differentiate *R. africae*.[17] However, *R. africae* could not be distinguished from *R. parkeri* using these primers.[18] The three sets of primers were synthesized by Bioprobe Systeme (Paris, France).

**Amplification by PCR.** The PCR amplification was carried out in 100-µl volumes using 10 µl of boiled sample and 2.5 units of *Taq* polymerase (Promega, Madison, WI) in a Prem III thermal cycler (LEP Scientific, Milton Keynes, UK) with 30 cycles of denaturation (20 sec at 95°C), annealing (30 sec at 48°C), and extension (2 min at 60°C). The reaction was boosted by adding another 2.5 units of *Taq* polymerase after 15 cycles. Three controls, two negative (distilled water and uninfected Vero cells) and one positive (*R. conorii* Moroccan strain), were included in each test.

**Digestion and electrophoresis of DNA.** The PCR amplification of DNA was verified by rapid agarose gel electrophoresis of 10 µl of PCR product and staining with ethidium bromide. Restriction endonuclease digestion was done with 50 µl of the PCR mixture using standard techniques.[19] The restriction enzymes chosen were *Alu* I for the *Rp* CS.877p-1258n amplification product, *Rsa* I and *Pst* I for the *Rr* 190.70p-602n amplification product, and *Rsa* I for the BG 1-2 amplification product. All endonucleases were obtained from New England Biolabs (Beverly, MA). Electrophoresis was conducted in 8% polyacrylamide vertical gels at 80 V for 5 hr. The DNA molecular weight marker V (Boehringer, Mannheim, Germany) was used as a source of DNA fragment size standards. The patterns obtained were compared with those previously obtained with the 16 reference strains of SFG rickettsiae that have been tested in our laboratory: *R. africae*, *R. akari*, *R. australis*, *R. bellii*, *R. conorii*, *R. helvetica*, *R. japonica*, *R. massiliae*, *R. montana*, *R. parkeri*, *R. rhipicephali*, *R. rickettsii*, *R. sibirica*, *R. slovaca*, the Itt rickettsia, and the Thai tick typhus rickettsia (Eremeeva M and others, unpublished data).

*Culture*

To confirm the PCR-RFLP identification of the different rickettsial species, we attempted to isolate the strains using the modified shell-vial centrifugation technique, as previously described.[8] Five different samples were used for each species identified. The second aliquot of each sample was thawed and inoculated into triplicate shell vials (3.7 ml; Sterilin, Feltham, UK) containing a monolayer of human embryonic lung fibroblasts grown on a coverslip 1 cm in diameter. After inoculation, the shell vials were centrifuged for 1 hr at 700 × *g* at 37°C. The inoculum was removed and replaced with culture medium (Eagle's minimal essential medium [MEM] containing 10% fetal bovine serum, 2 mM L-glutamine, 150 µg/ml of sulfamethoxazole, 200 µg/ml of gentamicin, and 0.1% amphotericin B). After incubation for five days at 37°C, a coverslip from one shell vial for each tick isolate was used for analysis by microimmunofluorescence using the method of Marrero and Raoult.[19] If dense rickettsial infections were present, the isolation procedure was regarded as successful. In such a case, the cells in the duplicate vial were passaged following trypsinization into a 25-cm$^2$ flask and incubated at 37°C in antibiotic-free MEM. Rickettsial infection was monitored by Gimenez staining of cells scraped from the bottom of the flasks.[20]

## RESULTS

Among the 310 tested specimens, 67 (21.6%) were amplified by the two first sets of primers.

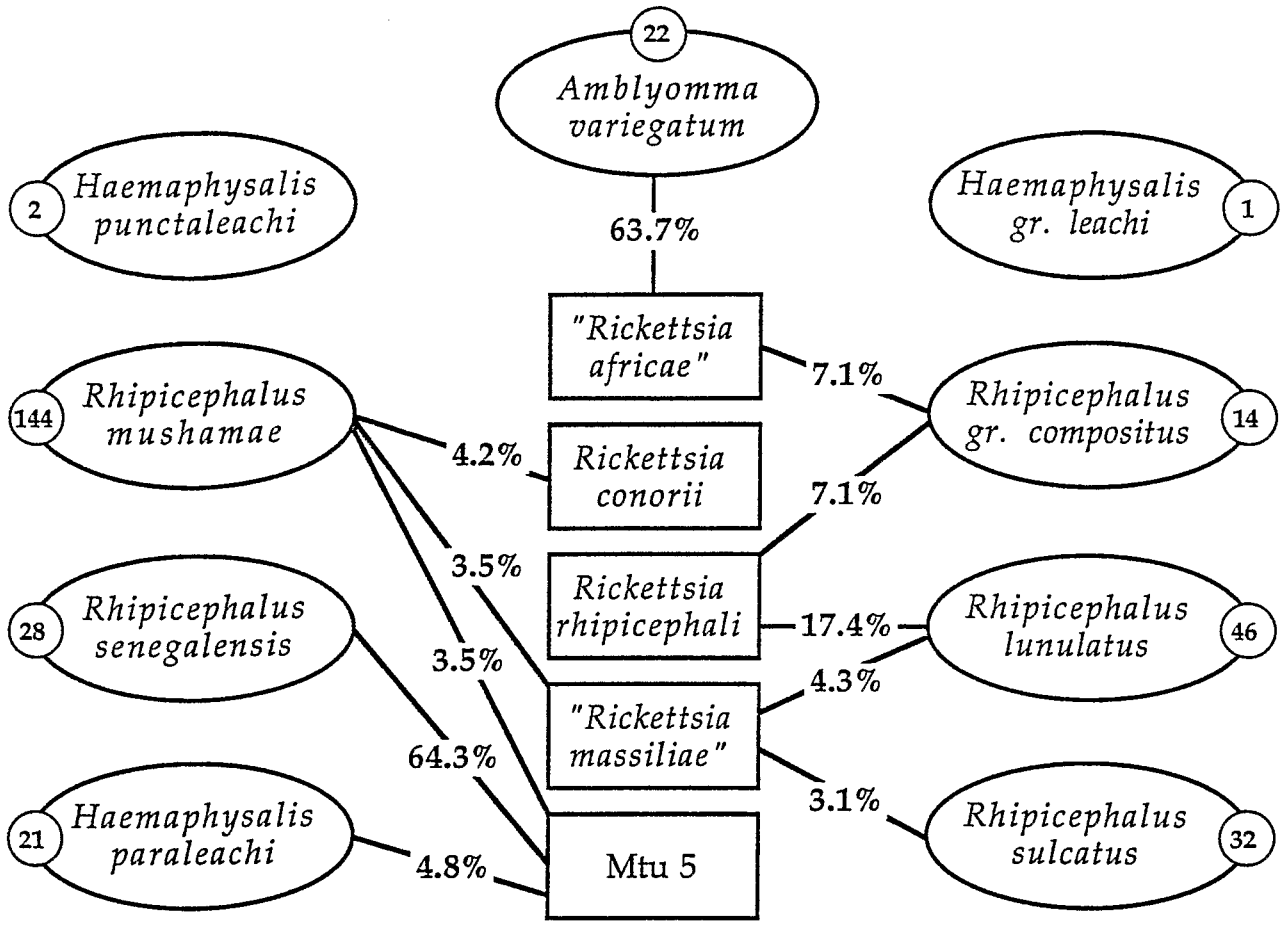


FIGURE 1. Tick infection by spotted fever group rickettsiae in the Central African Republic. Circled values are the number of ticks tested.

Fourteen of 22 *A. variegatum* (63.7%) were positive. Since all their restriction endonuclease patterns were identical to those of the Itt rickettsia, we further identified these specimens using *Rp* CS.877p-1258n and *Rr* 190.70p-602n. Using the BG 1-2 set of primers, the patterns obtained were in fact those of *R. africae* or *R. parkeri.*

Among the *Rhipicephalus* genus, the percent infected varied from 3.1% to 64.3%. Eighteen *Rh. senegalensis* (64.3%) were positive. All of them showed the same pattern that was identical to that of the Mtu 5 isolate found in *Rh. turanicus* in southern France.[21] Among the 13 positive *Rh. lunulatus,* eight (28.3%) showed a pattern identical with that of *R. rhipicephali,* and three were consistent with SFG rickettsia using the *Rp* CS.877p-1258n set of primers, whereas the *Rr* 190.70p-602n amplification was too weak for enzymatic restriction. Two specimens showed a profile identical to that of *R. massiliae* found in *Rh. turanicus* in southern France.[21] One sample showed only weak amplification using *Rp* CS.877p-1258n and was not considered positive.

Among the three positive *Rh. gr. compositus* (21.6%), one was consistent with SFG rickettsia using the *Rp* CS.877p-1258n set of primers, whereas the *Rr* 190.70p-602n amplification was too weak for enzymatic restriction. The second showed a profile consistent with *R. rhipicephali,* whereas the third showed a profile consistent with either Itt rickettsia or *R. africae* using *Rr* 190.70p-602n. The BG 1-2 amplification confirmed the identification of *R. africae* or *R. parkeri.* Seventeen *Rh. mushamae* (11.8%) were amplified using *Rp* CS.877p-1258n. The *Rr* 190.70p-602n amplification showed six profiles consistent with *R. conorii* strain 7, five consistent with *R. massiliae,* five consistent with Mtu5, and one required BG 1-2 amplification to show the presence of *R. africae* or *R. parkeri.* Six other samples of *Rh. mushamae* were weakly amplified only by *Rp* CS.877p-1258n and were not considered positive. The single positive *Rh. sulcatus* (3.1%) presented a profile consistent with *R. massiliae.*

Among the *Haemaphysalis* genus, a single *H.*

*paraleachi* (4.8%) was positive and showed a profile consistent with Mtu5. These results are summarized in Figure 1.

For each species identified, we attempted to isolate and cultivate the strain using the shell-vial centrifugation technique.[8] Although this technique has allowed us to cultivate rickettsiae from hundreds of various samples, we have not been able to isolate a single strain from our tick samples: most of them were contaminated despite the use of antibiotics and the other remained uninfected. This situation can be explained by the poor preservation conditions of our samples that had been collected six years before the study, kept at −80°C, but thawed at least three times.

## DISCUSSION

The epidemiology of SFG rickettsioses in Africa is largely unknown. This is due to the fact that few teams work on rickettsiosis epidemiology, and field methods are generally not available. A few articles, not recent, report the presence of *R. conorii* in *A. variegatum* in Senegal,[22] and in *Amblyomma, Hyalomma, Boophilus,* and *Rhipicephalus* in Africa.[23] Burgdorfer and others have also reported in 1973 the presence of a rickettsia closely related to *R. conorii* in *Amblyomma* ticks in Ethiopia.[24] It is identical to a rickettsia isolated from humans in Zimbabwe that we propose to call *R. africae,* the agent of African tick bite fever.[17]

These studies were conducted by staining crushed ticks with Machiavello red or by inoculation into guinea pigs and mice. The reference technique used to show the presence of rickettsiae in ticks is the hemolymph test. Isolation and identification were performed following inoculation into animals and clinical and serologic follow-up.

With the development of the shell-vial centrifugation technique,[25] we have a useful and reliable tool for isolation and identification of rickettsiae from ticks. Although this technique is useful for large-scale tick studies, it needs ticks to be alive for hemolymph inoculation or to be frozen at −80°C for crushed tick inoculation.[8] However, the main problem with these techniques in their application to African studies is the preservation and transportation of the samples.

For poorly preserved frozen samples such as ours, or when the ticks are fixed in alcohol or formaldehyde (tick collections), culture is impossible. In such cases, PCR-RFLP is a useful tool for the identification of the rickettsiae. This technique was first applied to DNA extracted from purified rickettsiae,[9] then to nonpurified rickettsiae in cell cultures.[26] Azad and others had applied the PCR-RFLP technique to the detection of rickettsiae in arthropod vectors (*R. typhi* in *Xenopsylla cheopis* fleas and *R. rickettsii* in *Dermacentor variabilis* ticks).[27] More recently, the same group has detected SFG rickettsiae in ticks from northern Sinai (Egypt) using concurrently PCR-RFLP and the hemolymph test.[13] Their study also confirms the validity of the technique. Another group has recently used the PCR to detect *R. rickettsii* in saliva, hemolymph, and triturated tissues of *D. andersoni,* as well as on fresh ticks and ethyl alcohol–preserved ones.[14] They were not able to detect rickettsiae in formaldehyde-preserved samples. However, we think this could be possible since Stein and Raoult have amplified *Coxiella burnetii* preserved in formaldehyde using Chelex 100* (Bio-Rad, Richmond, CA).[28]

In our experience, PCR-RFLP was easy to use and was reproducible after a few trials. As for time consumption, we were able to test our 310 samples within two months, i.e., approximately 40 samples per week. The cost of reagents was acceptable (between $3 and $4 U.S. dollars per sample). Besides the thermocycler, all the required equipment is standard in most laboratories. Moreover, although samples had been stored a long time (six years) and in uncertain conditions, we were able to detect rickettsiae in more than 20% of them, which is higher than commonly described. Materials contained in undigested blood meals (hemoglobin and hematin) are thought to be inhibitory in the PCR.[29] Although a large proportion of our ticks were engorged, we had no inhibition problems. Thus, the PCR-RFLP seems to be a useful tool for screening ticks to focus studies in areas where the prevalence of infection is high enough, and to select the tick species that will have to be harvested. These large screenings would be interesting to conduct on old tick collections, generally preserved in alcohol, which does not inhibit amplification.[14]

As for previous tick studies, Camicas published in 1975 a review of *Ixodidae* species in which *R. conorii* had been isolated in the Ethi-

opian and Mediterranean-European zoogeographic regions.[30] He mentioned the species we studied. In recent work, Lange and others studied *Rh. sanguineus* and three *Hyalomma* species that were different from those we studied.[13]

Our mean infection rate of ticks was 21.6% and ranged from 0% to 64.3%, depending on tick species. These results are consistent with those reported in Egypt (65–68% of *R. conorii*–infected ticks).[31] Burgdorfer and others[24] had found a lower percentage of infection (2.2%) of *Amblyomma* ticks by the rickettsia, which was recently named *R. africae*.[17,24] The only other African tick study was performed in Zimbabwe by Kelly and Mason.[32] They have shown the presence of different serovars of *R. conorii* by the hemolymph test and immunofluorescence. The infection rates were lower, ranging from 0% for *Rh. sanguineus* to 4% for *H. leachi* and 12% for *Rh. simus*.

All other studies conducted in other parts of the world also showed lower infection rates. In Europe in 1990, Peter and others used for the first time the shell-vial centrifugation technique on more than 500 *Rh. sanguineus* ticks from southern France.[25] The hemolymph test result was positive for 13.5% of the ticks and cell culture demonstrated the presence of *R. conorii* in 11.9%. In Sicily, Tringali and others have demonstrated the presence of *R. conorii* in 19.7% of *Rh. sanguineus,* ranging from 7.1% to 34.8%, depending on the sites.[33] In the United States, Philip and Casper demonstrated in California in 1981 the presence of *R. rickettsii* in *Dermacentor* and *Ixodes* ticks.[34] The infection rate was 19.4% (range 0–23%) using the hemolymph test and 9.7% using culture. Magnarelli and others conducted a similar study in Connecticut in 1981.[35] Using direct immunofluorescence, they showed that the infection rates were 1.8% for *D. variabilis* larvae, 7.8% for nymphs, and 40% for *I. cookei* nymphs. The hemolymph test showed that 8.8% of *D. variabilis* adults and 8.6% of *I. texanus* adults were positive. The same group conducted another study in 1985 that showed that 5.9% of the *D. variabilis* ticks were infected with *R. rickettsii* or *R. montana*.[36]

All the restriction endonuclease digestion profiles we produced were species-specific except for *R. africae,* which could not be distinguished from *R. parkeri.* Following the algorithm proposed by Beati and others,[21] *R. conorii* and *R. africae* (if the latter is really *R. africae* and not *R. parkeri*) showed known restriction profiles and were the expected strains in this area. In such conditions, we conclude that they are correctly identified. As for the other suspected species (*R. rhipicephali, R. massiliae,* and Mtu5), their restriction profiles are known and specific, but they have never been described in Africa. In such cases, the strains should be isolated, cultivated, and identified by serotyping with reference pool sera. Unfortunately, we have not been able to isolate these strains due to the age and the preservation conditions of the samples. Although we could not confirm the presence of these strains, we can conclude that five different SFG rickettsiae are prevalent in Central Africa.

The present study reveals a wide heterogeneity in tick infection by rickettsiae: the same tick species was parasitized by different rickettsiae, and the same rickettsiae species could be found in different tick species. This situation has been previously described. In the former USSR, *R. sibirica* has been isolated from four different *Dermacentor* species (*D. nuttalli, D. marginatus, D. silvarum,* and *D. reticulatus*),[37,38] but *R. slovaca* was also isolated from *D. marginatus*.[39] In the United States, *R. rickettsii, R. montana, R. parkeri,* and *R. bellii* have been found in various ticks (*D. andersoni, D. variabilis,* and *D. parumapertus*).[38,40,41] *Rhipicephalus sanguineus* can be infected by *R. rhipicephali,*[42] *R. rickettsii,*[43] *R. missiliae,*[44] and *R. conorii.* Moreover, isolating a rickettsia from a tick does not mean that this tick is capable of being a reservoir. To demonstrate the specificity of a tick-rickettsia relationship, it is necessary to show transovarial and a trans-stadial transmission of the rickettsia.

In a recent review, Brouqui and others reported the presence of rickettsiae throughout the African continent.[45] As for the intertropical area, numerous studies have shown the presence of rickettsiae in Cameroon,[3] the Congo,[46] Kenya,[47] Burkina Faso,[48,49] Rwanda and Burundi,[46] Ethiopia,[30,50] Zaire,[24] and Tanzania.[51] In Zimbabwe, Kelly and others found a human seroprevalence of 52% for *R. conorii* (Kenya strain) and 55% for the Zimbabwean SFG rickettsia.[52] Kelly and Mason also found antibodies to *R. conorii* among 82% of the dogs and 69% of the humans in another study.[32] In the same study, they reported a prevalence of tick infection by rickettsiae of 12% among *Rh. simus* and 4% among *H. leachi.* As for the Central African Republic, a serosurvey was conducted in 1985 in the village

of Bozo and the town of Bouar using indirect immunofluorescence.[2] Antibodies reactive with SFG rickettsiae were found in 59% of the inhabitants of Bouar and in 71% of the inhabitants of Bozo. This high human seroprevalence in Central Africa correlates well with the high rickettsial infection prevalence in ticks. Moreover, in the area studied, 95–97% of human tick bites are due to *A. variegatum* (Morel P, unpublished data). This correlates with our results showing 63.7% of these ticks infected by *R. africae*, the agent of tick bite fever.

In conclusion, we have used the PCR technique to conduct a preliminary study showing the presence of SFG rickettsiae in the Central African Republic. The association of high seroprevalence rates with a high level of tick infection by rickettsiae indicates that further studies are needed. These should be focused on the tick species that have shown the highest rates of infection with the rickettsiae that have never been described in Africa: *Rh. lunulatus* for *R. rhipicephali*, *Rh. senegalensis* for Mtu5, *Rhipicephalus* sp. for *R. massiliae*, and *A. variegatum* for *R. africae*. These ticks should be transported live to our laboratory so that rickettsiae can be isolated and identified by traditional procedures.

Acknowledgment: We thank Dr. Patrick Kelly for reviewing the manuscript.

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