DETECTION OF RICKETTSIA AFRICAEC IN PATIENTS AND TICKS ALONG THE COASTAL REGION OF CAMEROON

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Abstract. Rickettsia africae was identified in seven (6%) of 118 patients with acute fevers of unknown etiology proven not to be malaria or typhoid fever from clinics along the coastal region of Cameroon by polymerase chain reaction (PCR) amplification and sequencing of the citrate synthase (gltA) and outer membrane protein A (ompA) genes of Rickettsia. The majority (71%) of the patients were female. Clinical manifestations included fever (100%), headache (71%), myalgia (71%), arthralgia (43%), pulmonary involvement (29%), and diffuse rash (14%). Moreover, R. africae was detected by PCR amplification and sequence analysis of the gltA and ompA genes in 62 (75%) of 83 adult Amblyomma variegatum ticks collected from cattle in the same region. These results confirm the presence of a previously unrecognized infectious disease in the indigenous Cameroonian population, as well as extend the established range of R. africae.

INTRODUCTION

Spotted fever group (SFG) rickettsioses are caused by obligately intracellular gram-negative bacteria of the genus Rickettsia. Like other rickettsiae, transmission to humans occurs via arthropod vectors such as ticks. Although these infections have been recognized worldwide, their importance as a cause of febrile illness has been demonstrated serologically in Cameroonian patients only recently.1

In sub-Saharan Africa, one of the most important rickettsioses is African tick-bite fever (ATBF) caused by R. africae, with ticks of the genus Amblyomma serving as both vector and reservoir.2–7 The prevalence of rickettsial infection has been reported to be consistent with the geographic distribution of the host ticks Amblyomma hebraeum and A. variegatum that are widely distributed in sub-Saharan Africa (Figure 1).8–10

Since the first description of R. africae as a human pathogen in 1992, ATBF has been recognized as endemic in southern African countries such as Zimbabwe and South Africa.5 Serologic surveys in Angola, Burkina Faso, Central African Republic, Congo, Côte d’Ivoire, Mali, and Zimbabwe showed the presence of antibodies to SFG rickettsiae in healthy subjects in these populations.11,12 Recent serologic evidence has shown a high infection rate among indigenous populations in Cameroon in whom differential antibody titers and Western immunoblotting strongly supported the identification of R. africae as the cause of the illness in 26 of these indigenous African patients.1 Although serologic evidence supports a highly prevalent SFG rickettsiosis, only a single African patient has been documented to have ATBF by specific identification of the agent in the patient’s blood.3 Substantial information regarding the etiology and clinical manifestations of ATBF has been gathered from infected travelers returning to Europe and North America from Africa.13–17 Raoult and others reported SFG rickettsioses in travelers returning to Europe from Africa with documentation of R. africae by isolation of the agent and specific polymerase chain reaction (PCR) assays.13 However, the prevalence of ATBF among indigenous populations as well as the precise geographic distribution of R. africae remain largely unknown and warrant further epidemiologic studies on humans, mammals, and ticks in Africa.

To date, no studies have been conducted to detect potential tick vectors of rickettsiae in Cameroon. In this study, we used highly sensitive molecular-based assays to diagnose SFG rickettsiosis in patients who presented with symptoms of acute febrile illness at local clinics in the South West Province of Cameroon and whose laboratory test results for malaria and typhoid fever, the two known endemic fevers, were negative, and to determine the prevalence of rickettsiae in the suspected tick vector A. variegatum.

MATERIALS AND METHODS

Patient population. Blood (3 mL) was collected into sterile tubes containing EDTA from patients who presented with febrile illness at the Cameroon Development Corporation Central Clinic in Tiko and the Mount Mary Health Center in Buea, Cameroon between January and June 2003. Patient samples were routinely tested for detectable malaria parasites and for antibodies diagnostic of typhoid fever. Patient samples, which tested negative for both malaria and typhoid fever, were transported on ice to the Rickettsial Laboratory at the University of Buea for diagnosis of rickettsial infection. Whole blood was collected from 118 patients (77 females and 41 males). The patients came from different localities along the coast of Cameroon: Buea (4°9’N, 9°13’E), 29 patients; Limbe (4°2’N, 9°19’E), 38 patients; Muyuka (4°10’N, 9°25’E), 19 patients; and Tiko (4°2’N, 9°19’E), 32 patients. This research was conducted with approval according to the guidelines governing research at the clinical institutions from where patient samples were collected and at the University of Buea.

Tick collection. A total of 98 adult A. variegatum ticks (76 males and 22 females) were collected from cattle along the Atlantic Coast of Cameroon: Buea (4°9’N, 9°13’E), 46 ticks and Limbe (4°2’N, 9°19’E), 52 ticks. We selected these localities because we had previously demonstrated the presence of antibodies to SFG Rickettsia in febrile patients in this region.4 Collection of ticks from cattle was done with the permission of the owners, and care was taken to minimize discomfort for the animals. Collected ticks were stored in 1.5-mL vials containing 70% ethanol. Adult ticks identified using basic taxonomic schemes as A. variegatum were transported to the University of Texas Medical Branch at Galveston.18
Isolation of DNA from patients and ticks. The DNA was extracted from 50 μL of blood using the DNeasy Tissue Extraction Kit (Qiagen, Chatsworth, CA) following the manufacturer’s protocol. Purified DNA was quantified using a digital spectrophotometer at 260 nm wavelength (Perkin Elmer MLA 2000, Norwalk, CT) and stored at 4°C until used as template in the PCRs. Amblyomma variegatum were stored at 4°C until used for the isolation of genomic DNA. Ticks were surface sterilized by washing three times in 70% ethanol and stored in ethanol until DNA extraction. Before DNA extraction, ticks were each rinsed three times with sterile phosphate-buffered saline to remove any residual ethanol. A preliminary experiment to determine if ticks were infected with Rickettsia was carried out for five pools consisting of three male ticks each. Ticks in each pool were cut into small pieces with separate sterile scissors and homogenized with a separate sterile micropestle in a sterile 1.5-mL microtube. The DNA was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer’s instructions for isolation of DNA from animal tissues. Ticks were individually placed in a 1.5-mL microtube and rinsed as described earlier. Each tick was cut into small pieces with separate sterile scissors and homogenized with a separate sterile micropestle. The DNA was extracted as reported earlier, and quantified in a digital spectrophotometer at 260 nm wavelength (Perkin Elmer MLA 2000). Purified DNA was stored at 4°C until used as template for PCR amplifications.

Polymerase chain reaction amplification of rickettsiae from patient and tick samples. The PCRs were set up with approximately 250 ng of patient or tick DNA. All DNA samples extracted from the patients were individually processed by a real-time PCR assay with primers CS-5 (forward) and CS-6 (reverse) (Table 1) as previously described.19 The CS-78 and CS 323 primers that amplify the citrate synthase (gltA) gene were used to detect Rickettsia in the DNA extracted from the five pools of ticks as described earlier. Rickettsia was detected in all the pools; thus, we proceeded to extract DNA and amplify the gltA and outer membrane protein (ompA) genes from individual ticks. Patient and tick samples that had been amplified using primers CS-5 and CS-6 were examined again by PCR amplification using the primer pair CS 78 (forward) and CS-323 (reverse) and for ompA gene using the primers designed by Regnery and others20 (Table 1) to produce larger PCR amplicons for specific genetic identification of the infecting rickettsiae as described previously. Ten microliters of PCR product were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and examined using an ultraviolet transilluminator.

Sequencing of PCR products. The PCR products were rendered free of excess primers and nucleotides using EXOSAP-IT (United States Biochemical Corporation, Cleveland, OH) according to the manufacturer’s instructions. Cleaned products were sequenced directly using an ABI automated sequencer (Applied Biosystems, Foster City, CA). The BLAST program was used for sequence comparisons (National Center for Biotechnology Information, Bethesda, MD).

RESULTS

Rickettsia africæ infection in patients. Real-time PCR analysis showed that samples from seven patients contained DNA of the rickettsial gltA gene. Of the seven real-time PCR-positive patients, three were also positive by routine PCR using primers CS-78 and CS-323, which amplified a 401-basepair (bp) fragment of the gltA gene. The nucleotide sequences of these PCR products were 100% identical to R. africæ (GenBank accession number RAU59733). Four of the real-time PCR positive patients were also positive by routine PCR that amplified the ompA gene. The DNA sequences of the 5’ domain of ompA PCR products demonstrated 100% homology with R. africæ (GenBank accession number RAU43790). Three of the patients samples were positive for neither the gltA nor ompA genes by routine PCR. However, DNA sequence analysis of the real time PCR product (approximately 100 bp excluding the primer region) was 100% identical to R. africæ (RAU59733). These three samples had a very high critical threshold by real-time PCR, which suggested a very low rickettsial concentration. Five (7%) of the 69 female and 2 (4%) of the 49 male patients had R. africæ DNA in their blood. The mean age of the patients was 32.4 years, and the clinical manifestations were consistent with acute rickettsial infection (Table 2).

Infection of ticks by rickettsiae. Of the 83 adult A. variegatum ticks processed individually, 62 (75%) ticks were detected to contain Rickettsia by PCR using primers specific for the ompA gene and the 147-bp fragment of the gltA gene (Figure 2). Fifteen (68%) of the 22 female ticks and 47 (77%) of the 61 male ticks were shown to contain Rickettsia. The DNA sequences of the ompA product showed 100% homology with published sequences of R. africæ (RAU43790). Amplifications using the primers CS-78 and CS-323 detected

**Table 1**

<table>
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<tr>
<th>Primers</th>
<th>Nucleotide sequence</th>
<th>Reference</th>
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<tr>
<td>CS-5</td>
<td>5’-GAGAGAAAAATTATATATCCAAA-3’</td>
<td>19</td>
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<tr>
<td>CS-6</td>
<td>5’-AGGGTGCTTCGTGACATTCTT-3’</td>
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<tr>
<td>CS-78</td>
<td>5’-GCAAATATCGGTGAAGTGTA-3’</td>
<td>19</td>
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<tr>
<td>CS-323</td>
<td>5’-GCTTCTTAAATTTCAAAATTAC-3’</td>
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<tr>
<td>Rr190.70p</td>
<td>5’-ATTGCCGATATTCTTACAAA-3’</td>
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</tr>
<tr>
<td>Rr190.602n</td>
<td>5’-ATGGCAGCATTCGGTCCCCCT-3’</td>
<td></td>
</tr>
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The identification of DNA of *R. africae* in the blood of acutely ill febrile patients in Cameroonian clinics established the occurrence of African tick bite fever in indigenous patients in Cameroon, confirming our previous diagnosis of *R. africae* in 26 other febrile Cameroonian patients by differential immunofluorescent antibody titters and Western immunoblotting. These results emphasize the importance of African tick bite fever as a prevalent disease in an indigenous population. Indeed, on the basis of studies that have shown delayed development of antibodies and low sensitivity of PCR examination of blood for the diagnosis of African tick bite fever, the incidence of *R. africae* infection in patients in coastal Cameroon may be even higher than our serologic and PCR studies suggest. However, it is highly likely that CSS and CS6 primers used in the real time PCR assay, which has a limit of detection of one copy of *gltA*, are more sensitive than previous primer sets used in other studies. The increased sensitivity of this assay eliminates the need to use a nested PCR protocol, thus significantly decreasing the possibility of false-positive results as a result of contamination.

### DISCUSSION

The identification of DNA of *R. africae* in the blood of acutely ill febrile patients in Cameroonian clinics establishes the occurrence of African tick bite fever in indigenous patients in Cameroon, confirming our previous diagnosis of *R. africae* in 26 other febrile Cameroonian patients by differential immunofluorescent antibody titters and Western immunoblotting. These results emphasize the importance of African tick bite fever as a prevalent disease in an indigenous population. Indeed, on the basis of studies that have shown delayed development of antibodies and low sensitivity of PCR examination of blood for the diagnosis of African tick bite fever, the incidence of *R. africae* infection in patients in coastal Cameroon may be even higher than our serologic and PCR studies suggest. However, it is highly likely that CSS and CS6 primers used in the real time PCR assay, which has a limit of detection of one copy of *gltA*, are more sensitive than previous primer sets used in other studies. The increased sensitivity of this assay eliminates the need to use a nested PCR protocol, thus significantly decreasing the possibility of false-positive results as a result of contamination.

Knowledge of the clinical manifestations of African tick bite fever has been based on observations of European and North American travelers upon their return from rural southern Africa, one indigenous Zimbabwean patient, and our initial series of 75 Cameroonian patients with acute febrile illness leading to laboratory evaluation for malaria and typhoid fever. The returned travelers manifested fever (81–100%), headache (83–100%), myalgia (63–87%), eschar (53–100%), multiple eschars (21–83%), regional lymphadenitis (17–49%), lymphangitis (0–50%), rash (33–46%) that was in some cases maculopapular and others vesicular, and aphthous stomatitis (0–11%). The 36-year-old woman from Zimbabwe had fever, headache, and regional lymphadenopathy, but no rash or eschar. Our seven patients confirmed the occurrence of fever, headache, myalgia, and lack of prominence of rash (especially in darkly pigmented skin). Future clinical studies should include a careful examination for eschars, rash, regional lymphadenopathy, and aphthous stomatitis in African patients suspected of having African tick bite fever.

The identification of *R. africae* in *Amblyomma variegatum* ticks in Cameroon confirms the presence of the pathogen in the extended geographic distribution and indicates this species as the likely vector of *R. africae* in Cameroon. *Rickettsia africae* had now been identified in ticks or patients originating in Ethiopia, Zimbabwe, South Africa, Swaziland, Lesotho, Botswana, Tanzania, Kenya, Burundi, Mali, Niger, Gabon, Central African Republic, Sudan, Gambia, Ivory Coast, Cameroon, and Guadeloupe.

The prevalence of *R. africae* in 75% of *A. variegatum* ticks is similar to what has been observed in Zimbabwe, Central African Republic, Mali, and Niger. This extremely high prevalence is consistent with the occurrence of cases in clusters where groups of persons are exposed to a tick-infected environment. The occurrence of transovarian and transstadial maintenance of *R. africae* in *Amblyomma* ticks indicates the potential for transmission to humans by all stages of feeding ticks (larvae, nymphs, and adults). The high prevalence of this mildly pathogenic rickettsia in ticks contrasts dramatically with the low prevalence of *R. rickettsii* in *Dermacentor* ticks, which have the pathogenic effects of the highly virulent rickettsiae.
We did not identify any genotypic variation in these ticks, as was reported in ticks collected from Niger and Mali.\textsuperscript{7,10} This homogeneity may be attributable to the fact that our ticks were collected from two nearby locations: Buea and Limbe (Figure 1). Further analysis of \textit{A. variegatum} ticks from different regions may reveal genetic diversity of \textit{R. africae} in Cameroon. Variants of \textit{R. africae} have been detected in other tick species such as \textit{Rhipicephalus appendiculatus} and \textit{R. compositus}, although their role in the transmission of this infection has not been defined.\textsuperscript{4,7}

Although this study demonstrated \textit{R. africae} infection in febrile patients and ticks by PCR and DNA sequence analysis, the gold standard method to establish an etiologic agent is by isolation of the agent from the blood or tissue of suspected patients; this remains to be achieved in Cameroon. Other issues that should be addressed are a full description of the clinical spectrum of this rickettsiosis in African patients and the determination of risk factors for severe illness.

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