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Molecular and Clinical Evidence of *Ehrlichia chaffeensis* Infection in Cameroonian Patients with Undifferentiated Febrile Illness

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Abstract

Human monocytotropic ehrlichiosis (HME) is an emerging tick-transmitted zoonosis in the United States caused by *Ehrlichia chaffeensis*. *Ehrlichia canis*, *E. chaffeensis* and *E. ewingii* have recently been detected in dogs and *Rhipicephalus sanguineus* ticks from Cameroon; thus the potential exists for human infections. The objective of this study was to determine if *Ehrlichia* species were associated with acute fevers of unknown etiology in patients from the coastal region of Cameroon. *E. chaffeensis* was detected in peripheral blood from 12 (10%) of 118 patients using real-time polymerase chain reaction (PCR) amplification of the genus-specific disulfide bond (*dsb*) formation protein gene. Furthermore, DNA sequencing of PCR amplicons revealed that the *dsb* gene sequence was identical to *E. chaffeensis* (Arkansas strain). Patients with detectable *E. chaffeensis* DNA had clinical manifestations that included fever, headache, myalgia, arthralgia, pulmonary involvement, and diffuse rash.

INTRODUCTION

Human monocytotropic ehrlichiosis (HME) has emerged as an important tick-borne infectious disease in the United States after the discovery of *Ehrlichia chaffeensis*, an obligately intracellular gram-negative bacterium transmitted primarily by *Amblyomma americanum* ticks.^{1–3} *E. chaffeensis* was isolated in 1991,⁴ and since that time HME or evidence of *E. chaffeensis* has been reported in more than 30 states in the US,⁵ Africa,^{6,7} Israel,^{4,8,9} Latin America^{10,11} and Asia.^{12–14} A geographically limited serosurvey for

human ehrlichiosis in Africa suggests that human ehrlichiosis exists, but is an infrequent infection.^{6,7} Noteworthy is a serologically and clinically well documented case of HME acquired in Mali and diagnosed in the United States,⁷ which provides the strongest evidence that *E. chaffeensis* is circulating among yet to be determined reservoirs and vectors in Africa.

A. americanum is found only in the United States;¹⁵ however, *E. chaffeensis* DNA has been detected in other tick species such as *Dermacentor variabilis*, *Ixodes pacificus*, *A. testudinarium*, *Haemaphysalis longicornis* and *H. yengi*.^{16–20} suggesting that the agent is not exclusive to *A. americanum*. Most recently, *E. canis* and *E. ewingii*, were detected in Cameroonian dogs²¹ and in *Rhipicephalus sanguineus* ticks obtained from those dogs.²² *E. chaffeensis* has not been reported in *R. sanguineus* ticks in the United States, but *E. canis* and *E. ewingii* DNA has been detected in *R. sanguineus* ticks from Oklahoma.²³ Although *R. sanguineus* ticks rarely bite humans in the United States, two stages (larvae and nymph) of these ticks commonly bite humans in Africa and, therefore, may be an important vector in the region with the potential to transmit these zoonotic agents to humans.

In this study, we used a highly sensitive, genus-specific PCR assay to diagnose ehrlichiosis 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.

MATERIALS AND METHODS

Patient population

Peripheral blood (3 mL) was collected in sterile tubes containing anticoagulant (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 detection of 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 ehrlichial infection. Whole blood was collected from 118 patients (77 females and 41 males), and a recent medical history and observed clinical signs were recorded for each patient. Patients also voluntarily provided information on contact with tick-infested domesticated animals. The patients resided in 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.

Isolation of DNA from patients

DNA was extracted from 50 µL of whole 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 MBA 2000, Norwalk, CT) and stored at 4° C until used as template for PCR amplifications.

Real-time PCR Assay

DNA extracted from blood was quantitated by spectrophotometry (A_{260}) and 250 ng of each sample was added to individual reactions that included the *Ehrlichia* genus-specific primer pair Dsb-330 (forward) and Dsb-728 (reverse) that amplified a 409 bp of the *dsb* gene as previously described.²⁴ The amplification reaction, in a final volume of 25 µl, contained

12.5 µl of iQ SYBRGreen Supermix (Bio-Rad, Hercules, CA) and 0.5 µl of each primer at 20 µM (final concentration of 400 nM). PCR cycling conditions consisted of 95° C for 2 min and 50 cycles of 15 s at 95° C, 30 s at 58° C and 30 s at 72° C. In each set of reactions, *E. canis* genomic DNA was included with each run as a positive control in addition to negative control reactions without DNA template. PCR was performed in 96-well plates using an iCycler iQ multicolor real-time PCR detection system equipped with appropriate filter sets and analyzed with iQ software v 3.1 (BioRad Laboratories). Samples were considered positive and threshold cycle (C_T) was determined after it crossed the minimum threshold level above background fluorescence after baseline subtraction.

Confirmation of PCR amplicons and DNA sequencing

All PCR amplicons obtained from six patient blood samples in the present study were also confirmed by agarose (1.5%) gel electrophoresis and visualized with ethidium bromide using an ultraviolet transilluminator. PCR amplicons (409-bp) were purified using EXOSAP-IT (USB Corporation, Cleveland, Ohio) according to the manufacturer's instructions and sequenced directly with the same primers (Dsb-330 and Dsb-728) used for PCR on an ABI automated sequencer (UTMB Protein Chemistry Laboratory). The BLAST program (National Center for Biotechnology Information, Bethesda, MD) was used to compare *dsb* sequences in order to determine the species.

Immunofluorescent antibody assay

Patients that had detectable *E. chaffeensis* DNA were screened for antibodies against *E. chaffeensis* (Arkansas) antigen slides as previously described.²⁵ For screening, sera were diluted 1:64 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% Tween 20.

RESULTS

Characteristics of the patient population studied

The 118 patients (77 females and 41 males) whose blood samples did not contain a detectable level of malaria parasites or antibodies to *Salmonella* species were studied. Their ages ranged from 1 to 64 years (mean 26.2 years). All the patients (100%) included in this study had a fever with onset ranging from one to nine days (average of four days) duration prior to consultation and sample collection. A minority of patients (n=16, 14%) reported having taken antibiotics. Other clinical signs included headache 96 (81%), arthritis 52 (44%), myalgia 48 (41%), pulmonary involvement 36 (31%) and rash 19 (16%); seven patients required hospitalization. Forty-two (36%) patients reported owning dogs or having domesticated animals (goats and sheep) on their property, and 36 (31%) confirmed that their animals were tick infested.

Characteristics of patients in whom *Ehrlichia* was detected by real-time PCR

PCR amplification of patient peripheral blood samples detected ehrlichial DNA in 12 (10%) of the 118 patients (6 females and 6 males). One patient was an infant (1 year-old), but the mean age of the patients was 23 years-old. Five patients were from Buea, three from Tiko and two each from Limbe and Muyuka. Clinical symptoms, in addition to fever, included headache (67%), myalgia (42%), arthralgia (58%), pulmonary involvement (17%) and rash (17%) (Table 1). None of the patients with ehrlichial DNA had any antibiotic therapy, and only one required hospitalization. Nine of the patients reared domesticated animals, and eight had contact with tick-infested animals.

PCR amplicon sequencing

Agarose gel electrophoresis of all the above samples that were detected by real-time PCR revealed a single band corresponding to the expected amplicon size (409 bp) (Fig. 1). Sequenced amplicons were 100% identical to each other and to the corresponding sequence of the United States isolates of *E. chaffeensis* (Arkansas) (GenBank accession numbers CP000236 and AF403711). Sequences from *E. chaffeensis dsb* PCR amplicons obtained from Cameroonian patients were deposited into GenBank and assigned accession numbers (EF375885-EF375887).

Immunofluorescent antibody assay

Patients that had detectable *E. chaffeensis* DNA did not have detectable antibodies to *E. chaffeensis* as determined by IFA.

DISCUSSION

This study is the first to provide molecular and clinical evidence of *E. chaffeensis* infection in a well characterized patient group with undifferentiated febrile illness in Africa. Although HME has been recognized as an important emerging disease causing extensive morbidity among humans in the United States, the geographic distribution of the disease remains undefined due to its recent emergence, challenges in diagnosing the disease, and lack of comprehensive epidemiological studies outside the United States. HME is difficult to diagnose because the clinical findings, which include fever, malaise, headache, myalgia, rigors, cough and rash are nonspecific, serology is often negative at the time of presentation, and isolation of the etiologic agent is difficult and requires weeks to obtain a result.²⁶ These HME patients reported symptoms and presented with clinical signs consistent with HME infection. Although molecular evidence of *E. chaffeensis* infection was demonstrated in these patients, we did not attempt to isolate the agent from blood or tissue of the suspected patients, nor were additional samples collected after convalescence to confirm infection serologically. However, at the time of presentation (average 4 days after onset of symptoms) antibodies were not present, therefore detection of the agent by PCR provided the best opportunity to identify *E. chaffeensis*-infected individuals. Molecular detection of *E. chaffeensis* appears to be most sensitive in HME patients in the acute phase at the time of presentation.²⁷ Nevertheless, future investigations including serologic evaluation and attempts to isolate the agent would be useful towards full characterization the *E. chaffeensis* strain(s) infecting patients in this country.

The *E. chaffeensis dsb* gene amplified from the blood of these febrile patients was identical to the gene sequence from US isolates. The homology observed between the *E. chaffeensis* strains from Cameroon and the United States suggests a common origin or introduction of the pathogen perhaps from imported dogs originating from the United States; however, it may also be endemic to this region, but unrecognized. Previously, we reported that the sequence of *dsb* genes of *E. canis* from dog blood and/or *R. sanguineus* ticks from Cameroon were identical to the isolates from the United States.^{19,20,25} Although the Cameroonian *E. canis* and North American *E. canis dsb* genes were identical, Cameroonian ehrlichiae could be distinguished molecularly based on the number of repeat units of the gp36 gene.²⁸ This observation indicates that the *dsb* gene is conserved in geographically dispersed isolates and characterization of additional genes that exhibit more diversity, such as VLPT, would potentially identify *E. chaffeensis* genotypes in Cameroon that differ from genotypes present in the United States.

HME manifests as an undifferentiated flu-like febrile illness, which is not easily clinically differentiated from many other febrile tropical diseases. Currently, diagnosis of HME is still

largely based on the combined evaluation of clinical signs, laboratory and epidemiological data. Since most physicians are unfamiliar with HME, this disease is often misdiagnosed and underdiagnosed even in the United States.^{29,30} The previous lack of documented HME cases in Cameroon may be due to the fact that it is generally considered a pathogen confined to North America and has been rarely diagnosed outside the United States. Furthermore, tick-borne diseases are not routinely considered in a differential diagnosis by local clinicians whose primary diagnostic focus is on endemic malaria and typhoid fever. This situation is complicated by the fact that the diagnosis typically requires specialized skills and equipment which are unavailable in local clinics. However, tick exposure in Cameroon is high and is associated with recreation, occupational, and peridomestic activities. A recent survey of febrile patients whose serum samples did not contain antibodies against *Salmonella typhi* and did not contain blood malarial parasites revealed a high prevalence of antibodies to *Rickettsia*, and subsequent molecular studies identified *R. africae* in the blood of seven of the 118 patients reported in this study.^{31,32} Notably, *E. chaffeensis* was not detected in the blood of any of the patients who were infected with *R. africae*.³² The difficulty in clinical discrimination between these tick-borne bacterial diseases and that caused by the hyperendemic *Plasmodium falciparum* may also contribute to the lack of recognition of these diseases.

The clinical course of this and other tick-borne diseases requires further investigation especially in sub-Saharan African countries where the prevalence of human immunodeficiency virus (HIV) infection is high. Severe manifestations of HME have been reported to occur more frequently among patients infected with HIV.³³ Thus, ehrlichiosis may be a life-threatening illness in HIV-infected persons. Although this study gives conclusive evidence of HME infection in Cameroon, other issues that should be addressed are a full description of the clinical spectrum of ehrlichiosis in African patients, determination of risk factors for severe illness, and isolation of the pathogen.

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FIGURE 1.

Agarose (1.5%) gel electrophoresis of *Ehrlichia dsb* gene amplicons (409-bp) from Cameroonian patient blood samples (9 of 12 patients). (Lanes 1 and 9), 100-bp molecular weight marker; (lanes 2 and 10), positive control, *E. canis* genomic DNA; (lanes 3 and 11), no DNA template controls; (lanes 4 – 8, 12–16), Cameroonian human patient samples.

Table 1

Epidemiologic and clinical characteristics of twelve Cameroonian patients with HME.

Patients	1	2	3	4	5	6	7	8	9	10	11	12
Gender	F	F	M	F	M	M	F	M	F	F	M	M
Age	63	40	5	23	22	20	21	1	16	26	35	25
Location	Buea	Limbe	Tiko	Muyuka	Tiko	Muyuka	Buea	Tiko	Buea	Buea	Limbe	Buea
Clinical Manifestations												
Temperature (°C)	39	38.5	38	38	38.5	38.5	39	39	38.5	38	38.5	39
Day(s) after onset (before collection of sample)	6	8	4	7	4	3	1	1	2	7	2	2
Headache	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes	Yes	No
Myalgia	Yes	Yes	No	No	No	Yes	No	No	Yes	No	Yes	No
Arthralgia	Yes	Yes	No	No	No	Yes	Yes	No	Yes	Yes	Yes	No
Rash	No	No	No	Yes	No	No	No	Yes	No	No	No	No

(arms)