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Predominance of *Ehrlichia chaffeensis* in *Rhipicephalus sanguineus* ticks from a kennel-confined dogs in Limbe, Cameroon

Lucy M. Ndip[1], **Roland N. Ndip**[2], **Seraphine N. Esemu**[1], **David H. Walker**[3,4], and **Jere W. McBride**[3,4,5]

[1] Department of Biochemistry and Microbiology, University of Buea, Buea, Cameroon

[2] Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa

[3] Department of Pathology, University of Texas Medical Branch Galveston, TX 77555-0609

[4] Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch Galveston, TX 77555-0609

[5] Department of Microbiology and Immunology, University of Texas Medical Branch Galveston, TX 77555-0609

Abstract

Rhipicephalus sanguineus ticks (n=63) collected from five dogs (two adults and three puppies) housed in a kennel were screened for ehrlichial agents (*E. canis*, *E. chaffeensis* and *E. ewingii*) using a species-specific multicolor real-time TaqMan PCR amplification of the (disulphide bond formation protein (*dsb)* gene. *E. chaffeensis* DNA was detected in 33 (56 %) ticks, *E. canis* DNA was detected in four (6 %) ticks, and one tick was coinfected. The *E. chaffeensis* and *E. canis* nucleotide sequences of the amplified *dsb* gene (374-bp) obtained from the Cameroonian *R. sanguineus* ticks were identical to the North American genotypes.

Keywords

R. sanguineus; *Ehrlichia chaffeensis*; *Ehrlichia canis*; Zoonosis; Cameroon

Introduction

Ehrlichiae are obligately intracellular gram-negative tick-transmitted bacteria that primarily infect monocytes or granulocytes and are responsible for diseases of human and veterinary importance worldwide (Maeda et al. 1987; Anderson et al. 1991; Buller et al. 1999). Agents such as *Ehrlichia chaffeensis* and *E. ewingii*, which cause human infections of varying severity, are considered to be emerging tick-borne zoonoses transmitted by *Amblyomma americanum* (Walker 1998). The diagnosis of these infections is largely based on the combined evaluation of clinical signs and laboratory and epidemiological data (Olano et al. 2003). Unfortunately, most physicians are unfamiliar with human ehrlichiosis, and the disease is not routinely considered in the clinical and laboratory diagnosis of undifferentiated flu-like febrile illness, particularly outside the known geographical distribution of the primary vector, *A. americanum.*

Correspondence: Lucy M. Ndip, Ph.D, Department of Biochemistry and Microbiology, University of Buea, P. O. Box 63, Buea, Cameroon. Tel: 237-9952-0459, Fax: 237-3332-3125.

E. chaffeensis and *E. ewingii* are two pathogens previously considered to be geographically limited to North America. However, the detection of *E. chaffeensis* reactive sera from humans and animals in other parts of the world such as Brazil, Mexico and Korea, where *A. americanum*, is not found suggests that *E. chaffeensis* may be prevalent in other geographical regions (Heppner et al. 1997; Gongora-Bianchi et al. 1999; Machado et al. 1999; Heo et al. 2002; Kim et al. 2003; Calic et al. 2004), and the agent has been detected in other tick species such as *Haemaphysalis longicornis* and *Ixodes persulcatus* (Cao et al. 2000; Lee et al. 2005). *E. canis* and *E. ewingii* DNA has been detected in dogs presenting at local veterinary clinics in Cameroon, indicating that multiple *Ehrlichia* species are present and suggesting that a tick vector other than *A. americanum* is responsible for transmission (Ndip et al. 2005). Moreover, DNA from multiple *Ehrlichia* species (*E. canis*, *E. ewingii* and *E. chaffeensis*) were detected in unengorged *R. sanguineus* ticks obtained from these dogs (Ndip et al. 2007). Findings reported by others, also suggests that the agents of human ehrlichiosis are present on the African continent. A serologically confirmed case of *E. chaffeensis* infection acquired in Mali has been reported (Uhaa et al. 1996). Furthermore, dogs examined in South Africa had antibody titers to *E. chaffeensis* higher than that for *E. canis*, suggesting that *E. chaffeensis* actually elicited the antibody response (Pretorius and Kelly, 1998). Others have suggested that human ehrlichial infections occur infrequently in the African continent (Brouqui et al. 1994), but the existence and epidemiology of human ehrlichioses in Africa are still undetermined.

The recent emergence and increased recognition of diseases caused by tick-transmitted ehrlichiae and the recent discoveries of rickettsial species in areas and of tick species that were previously thought to be uninfected by these agents have suggested that these agents may have wider distribution than the United States. This stimulated our interest to investigate the presence of these obligately intracellular bacteria in Cameroon. We report herein, a high *E. chaffeensis* prevalence in *R. sanguineus* ticks collected during a field collection from a group of kennel-confined dogs in Limbe, Cameroon.

Materials and Methods

Tick collection

A total of 63 (21 from dog 1; 14 from dog 2; eight from dog 3; eleven from dog 4; nine from dog 5) adult incompletely engorged *R. sanguineus* ticks were collected from five heavily infested dogs (two adults [dogs 1 and 2] and three juveniles [dogs 3–5]) in a kennel from the Atlantic coastal city of Limbe (4°2′N, 9°19′E), Cameroon. Consent to collect blood from the dogs was denied by the owner. Care was taken to minimize discomfort to the animals. Ticks were surface sterilized by washing three times in 70% ethanol, stored in 1.5-mL vials containing the same concentration of ethanol at 4°C until transported to University of Texas Medical Branch, Galveston, TX.

Isolation of DNA from ticks

Before DNA extraction, ticks were each rinsed three times with sterile phosphate-buffered saline (PBS) to remove any residual ethanol. Ticks 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. The DNA was quantified in a digital spectrophotometer at 260 nm wavelength (Perkin Elmer MBA 2000). Purified DNA was stored at 4°C until used as template for PCR amplifications.

PCR detection of Ehrlichia species in ticks

All ticks were individually processed and examined by multicolor realtime PCR to detect the presence of *E. canis*, *E. chaffeensis* or *E. ewingii* DNA in ticks. DNA (~250 ng) from each tick

was added to individual reactions (25μL) and the PCR product (378 bp) amplified using conditions, primers, and probes as previously described (Doyle et al. 2005). Plasmids containing the *dsb* gene of *E. chaffeensis*, *E. canis* or *E. ewingii* were included with each run as positive controls 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 the appropriate filter sets and analyzed with iQ software v 3.1 (BioRad Laboratories).

DNA sequencing

PCR amplicons were purified using EXOSAP-IT (USB Corporation, Cleveland, Ohio) according to the manufacturer's instructions and sequenced directly with the same primers 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 and genotype.

RESULTS

Detection of Ehrlichia DNA

Real-time PCR detected ehrlichial DNA in 38 (60 %) of the 63 *R. sanguineus* ticks. Real-time PCR with specific probes to detect *E. chaffeensis*, *E. canis* and *E. ewingii* confirmed the high prevalence of *E. chaffeensis* DNA in the tick samples. *E. chaffeensis* was detected in 33 (56 %) ticks, *E. canis* DNA was detected in four (6 %) ticks, and one tick was coinfected. *E. ewingii* DNA was not detected in this group of ticks. Amplicons (378 bp) amplified and identified by real-time PCR as *E. canis* or *E. chaffeensis* were sequenced and found to be identical to North American strains AF403710 and AY403711, respectively.

DISCUSSION

E. chaffeensis is a gram-negative bacterium that infects monocytes causing the zoonosis, human monocytic ehrlichiosis (HME), an important emerging tick-borne disease that was first reported in the United States in 1987 (Maeda et al. 1987; Anderson et al. 1991). Since then, many cases have been reported to the Centers for Diseases Control, and the number of cases diagnosed each year has risen (Paddock and Childs, 2003). In this study, we identified two ehrlichial species in these ticks, *E. chaffeensis* (56 %) and *E. canis* (6 %). While *R. sanguineus* is the known vector of *E. canis* worldwide, previous studies have demonstrated that *E. chaffeensis* is maintained in nature through a cycle involving white-tailed deer (*Odocoileus virginianus*) as the cardinal reservoir host and the lone star tick, *A. americanum* (Ewing et al. 1995; Anderson et al. 1991; Lockhart et al. 1997). However, our findings of a high of prevalence of *E. chaffeensis* in *R. sanguineus* ticks collected dogs in one kennel suggests that this pathogen not only circulates in Cameroon, but also that other tick vectors and/or reservoirs may be implicated in its transmission in other parts of the world where *A. americanum* is not present. This study adds to the increasing evidence that the pathogen is not geographically restricted to the continental United States. Growing evidence of *E. chaffeensis* infection in humans and other mammalian hosts such as dogs and deer in other continents is extending the known range of *E. chaffeensis* (Chahan et al. 2005; Heppner et al. 1997; Gongora-Bianchi et al. 1999; Machado et al. 1999; Heo et al. 2002; Kim et al. 2003; Calic et al. 2004). *E. chaffeensis* has also been detected in other tick species such *A. testudinarium* ticks from southern China (Cao et al. 2000) and in *H. longicornis* ticks from Korea (Lee et al. 2005) although their role in transmission of the pathogen has not been investigated.

This study identified *R. sanguineus* as a probable vector of *E. chaffeensis* in Cameroon. Recently, we examined 92 *R. sanguineus* ticks collected from 51 dogs from different sites in the Mount Cameroon region and found that these ticks were infected with three ehrlichial species, *E. canis*, *E. chaffeensis* and *E. ewingii*, the most prevalent being *E. canis* (Ndip et al. 2007). However, the present finding of a high prevalence of *E. chaffeensis* in ticks collected from dogs from one kennel suggests that *E. chaffeensis* has increased transmission efficiency where infection and reinfection potential is higher. A high number of *E. chaffeensis* infections and coinfections in kennel-confined dogs where *R. sanguineus* was the predominant tick vector has been reported, but the *Ehrlichia* species in these ticks was not determined (Kordick et al. 1999).

R. sanguineus are also important vectors of other tick-borne human pathogens, notably *R. conorii*, *R. rickettsii* (Mexico and Arizona) and *E. canis* and *Babesia canis*, the later three being pathogens of veterinary importance (Walker et al. 2003). These have three developmental stages, with the larvae and nymphal stages capable of feeding on humans (Walker et al. 2003). Therefore, the high prevalence of *E. chaffeensis* observed in these ticks suggests that this vector could potentially transmit this agent to humans in this region. The new discoveries of rickettsial agents in unexpected tick vectors seem to be on the rise. Recently, *R. rickettsii*, the agent of Rocky Mountain spotted fever known to be transmitted by *Dermacentor* spp., was detected in *R. sanguineus* ticks in Arizona (Demma et al. 2005).

Based on these findings, it is most likely that the exact distribution of *E. chaffeensis* and its vectors outside North America is not yet understood and warrants further investigation. In Cameroon, efforts will be concentrated on isolating ehrlichiae from suspected tick vectors and vertebrate hosts since isolation is the gold standard for diagnosis of any infectious disease and comprehensive characterization of these pathogens can only be done upon isolated organisms. Due to the potential health risk to the human population, it is also critical to examine the role of these pathogens in undifferentiated febrile illness. Although *E. chaffeensis* is not yet recognized as a problem in the tropics, the situation is likely to change if greater attention is paid to the possibility.

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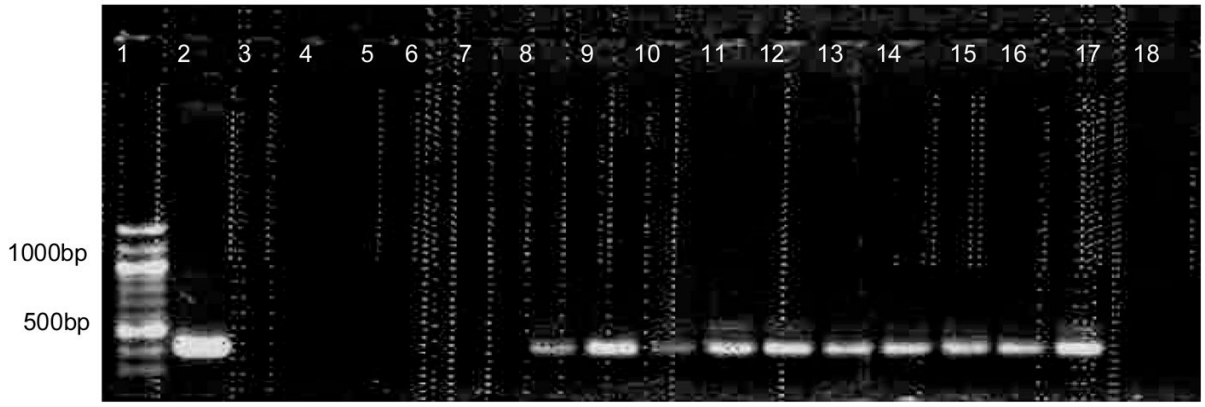

FIGURE 1.

Polymerase chain reaction–amplified products from *Rhipicephalus sanguineus* ticks. Amplification of a 378-basepair (bp) product of the dsb protein gene and electrophoresis on a 1.5% agarose gel. **B** Lane 1, 100-bp molecular weight marker; lane 2, positive control *E. canis* plasmid DNA; lanes 3 and 4, negative controls; lanes 5–7 and 18, negative samples of DNA extracted from *R. sanguineus* ticks, lanes 8–17, positive samples of DNA extracted from individual *R. sanguineus* ticks.