

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

LUCY M. NDIP, ERIC B. FOKAM, DONALD H. BOUYER, ROLAND N. NDIP, VINCENT P. K. TITANJI,
DAVID H. WALKER, AND JERE W. McBRIDE

Department of Life Sciences, University of Buea, Buea, South West Province, Cameroon; Department of Pathology and Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, Texas

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.³ 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.¹ 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

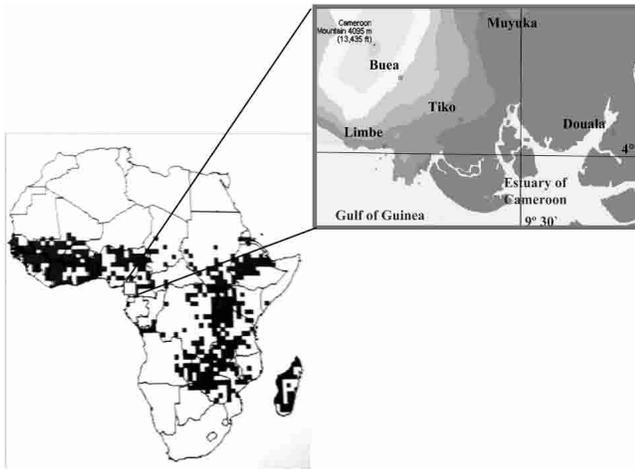


FIGURE 1. Distribution of *Amblyomma variegatum*, the vector of *Rickettsia africae* (lower left) and location of patients diagnosed with African tick-bite fever in Cameroon (upper right).

Isolation of DNA from patients and ticks. The DNA was extracted from 50 μ L 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 MBA 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 MBA 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)

TABLE 1
Primers and sequences used in this study

Primers	Nucleotide sequence	Reference
CS-5	5'-GAGAGAAAATTATATATCCAAA-3'	19
CS-6	5'-AGGGTCTTCGTGCATTCTT-3'	
CS-78	5'-GCAAGTATCGGTGAGGATGTA-3'	19
CS-323	5'-GCTTCCTTAAAATTCAATAAATC-3'	
Rr190.70p	5'-ATGGCGAATATTTCTCCAAAA-3'	20
Rr190.602n	5'-AGTGCAGCATTCGCTCCCCCT-3'	

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 africae* 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. africae* (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. africae* (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. africae* (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. africae* 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. africae* (RAU43790). Amplifications using the primers CS-78 and CS-323 detected

TABLE 2
Epidemiologic and clinical characteristics of seven Cameroonian patients with African tick-bite fever

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Epidemiologic data							
Sex	F	F	M	F	F	F	M
Age (years)	27	32	49	24	28	29	38
Location	Tiko	Limbe	Tiko	Buea	Buea	Limbe	Muyuka
Clinical characteristics							
Fever >38°C	Yes						
Days after onset sample collected	2	3	3	1	2	4	3
Headache	No	Yes	Yes	Yes	Yes	No	Yes
Myalgia	Yes	Yes	Yes	No	No	Yes	Yes
Arthralgia	Yes	No	No	Yes	No	No	Yes
Rash	No	No	No	No	No	Yes	No
Pulmonary signs	No	Yes	No	No	No	No	Yes

Rickettsia in 59 (15 female and 44 male) ticks. The sequences of the PCR products were identical to those reported for *R. africanae*.¹⁰

DISCUSSION

The identification of DNA of *R. africanae* 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. africanae* in 26 other febrile Cameroonian patients by differential immunofluorescent antibody titers 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. africanae* infection in patients in coastal Cameroon may be even higher than our serologic and PCR studies suggest.^{13,21} However, it is highly likely that CS5 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.¹⁹ 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. africanae* in *A. variegatum* ticks in Cameroon confirms the presence of the pathogen in the extended geographic distribution and indicts this species as the likely vector of *R. africanae* in Cameroon. *Rickettsia africanae* 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. africanae* in 75% of *A. variegatum* ticks is similar to what has been observed in Zimbabwe, Central African Republic, Mali, and Niger.^{4,5,10} This extremely high prevalence is consistent with the occurrence of cases in clusters where groups of persons are exposed to a tick-infected environment.^{13,16,17,22} The occurrence of transovarian and transstadial maintenance of *R. africanae* in *Amblyomma* ticks indicates the potential for transmission to humans by all stages of feeding ticks (larvae, nymphs, and adults).² 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.²³

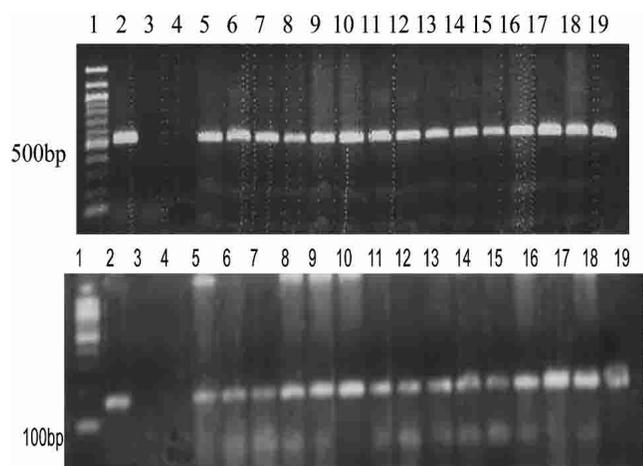


FIGURE 2. Polymerase chain reaction–amplified products from *Amblyomma variegatum* ticks. **Top**, Amplification of a 530-basepair (bp) product of the outer membrane protein A gene and electrophoresis on a 1.5% agarose gel. **Bottom**, Products amplified with the CS-5 and CS-6 primers and electrophoresis on a 3% Metaphor® (Cambrex, Walkersville, MD) agarose gel. Lane 1, 100-bp molecular weight marker; lane 2, positive control DNA extracted from Vero cells infected with *Rickettsia amblyommii*; lanes 3 and 4, negative controls; lanes 5–19, DNA extracted from individual *A. variegatum* ticks.

We did not identify any genotypic variation in these ticks, as was reported in ticks collected from Niger and Mali.^{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 *A. variegatum* ticks from different regions may reveal genetic diversity of *R. africanae* in Cameroon. Variants of *R. africanae* have been detected in other tick species such as *Rhipicephalus appendiculatus* and *R. compositus*, although their role in the transmission of this infection has not been defined.^{4,7}

Although this study demonstrated *R. africanae* 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.

Received April 5, 2004. Accepted for publication April 9, 2004.

Acknowledgment: We thank Dr. Marcelo Labruna for his assistance during this project.

Financial support: This research was supported by a National Institutes of Health training grant from the Fogarty International Center (D43-TW00903) to David H. Walker and a University of Buea Staff development grant to Lucy M. Ndip.

Authors' addresses: Lucy M. Ndip, Eric B. Fokam, Roland N. Ndip, and Vincent P. K. Titanji, Department of Life Sciences, Faculty of Science, University of Buea, PO Box 63, Buea, South West Province, Cameroon. Donald H. Bouyer, David H. Walker, and Jere W. McBride, Department of Pathology and Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, TX 77555-0609.

Reprints requests: David H. Walker, Department of Pathology, Director, World Health Organization Collaborating Center for Tropical Diseases, University of Texas Medical Branch Galveston, TX 77555-0609, Telephone: 409-772-2856, Fax: 409-772-1850, E-mail: dwalker@utmb.edu.

REFERENCES

- Ndip LM, Bouyer DH, Travassos da Rosa APA, Titanji VPK, Tesh RB, Walker DH, 2004. Acute spotted fever rickettsiosis among febrile patients, Cameroon. *Emerg Infect Dis* 10: 432–437.
- Kelly PJ, Mason PR, 1991. Transmission of spotted fever group rickettsia by *Amblyomma hebraeum* (Acari:Ixodidae). *J Med Entomol* 28: 598–600.
- Kelly P, Mathewman L, Beati L, 1992. African tick-bite fever: a new spotted fever group rickettsiosis under an old name. *Lancet* 340: 982–983.
- Dupont HT, Cornet JP, Raoult D, 1994. Identification of rickettsiae from ticks collected in the Central African Republic using the polymerase chain reaction. *Am J Trop Med Hyg* 50: 373–380.
- Beati L, Kelly PJ, Mathewman LA, Mason PR, Raoult D, 1995. Prevalence of rickettsia-like organisms and spotted fever group rickettsia in ticks (Acari : Ixodidae) from Zimbabwe. *J Med Entomol* 32: 787–792.
- Kelly PJ, Beati L, Mason PR, Mathewman LA, Roux V, Raoult D, 1996. *Rickettsia africanae* sp. nov., the aetiological agent of African tick-bite fever. *Int J Syst Bacteriol* 46: 611–614.
- Macaluso KR, Davis J, Alam U, Korman A, Rutherford JS, Rosenberg R, Azad A, 2003. Spotted fever group rickettsiae in ticks from the Masai Mara Region of Kenya. *Am J Trop Med Hyg* 68: 551–553.
- Estrada-Pena A, Jongejan F, 1999. Ticks feeding on humans: a review of records on human-biting ixodoidea with special reference to pathogen transmission. *Exp App Acarol* 23: 685–715.
- Beninati T, Lo N, Noda H, Esposito F, Rizzoli A, Favia G, Genchi C, 2002. First detection of spotted fever group rickettsiae in *Ixodes ricinus* from Italy. *Emerg Infect Dis* 8: 983–986.
- Parola P, Inokuma H, Camcas JL, Brouqui P, Raoult D, 2001. Detection and identification of spotted fever group rickettsiae and ehrlichiae in African ticks. *Emerg Infect Dis* 7: 1014–1017.
- Gonzales JP, Fiset P, George AJ, Saluzzo JR, Wiseman JR, 1985. Approche sérologique sur l'incidence des rickettsioses en République Centrafricaine. *Bull Soc Pathol Exot* 78: 153–156.
- Dupont HT, Brouqui P, Faugere B, Raoult D, 1995. Prevalence of antibodies to *Coxiella burnetii*, *Rickettsia conorii*, and *Rickettsia typhi* in seven African countries. *Clin Infect Dis* 21: 1126–1133.
- Raoult D, Fournier PE, Fenollar F, Jensionsius M, Prioe T, De Pina J, Caruso G, Jones N, Laferl H, Rosenblatt JE, Marrie TJ, 2001. *Rickettsia africanae*, a tick-borne pathogen in travelers to sub-Saharan Africa. *N Engl J Med* 334: 1504–1510.
- Kimura M, Fujii T, Iwamoto A, 1998. Two cases of spotted fever group rickettsiosis contracted in southern parts of Africa. *Kansenshogaku Zasshi* 72: 1311–1316.
- Jensionsius M, Hassle G, Henriksen AZ, Vene S, Raoult D, Bruu AL, Myrvang B, 1999. African tick-bite fever imported into Norway: presentation of 8 cases. *Scand J Infect Dis* 31: 131–133.
- Caruso G, Zasio C, Guzzo F, Granta C, Mondardini V, Guerra E, Macri E, Benedetti P, 2002. Outbreak of African tick-bite fever in six Italian tourists returning from South Africa. *Eur J Clin Microbiol Infect Dis* 21: 133–136.
- Jensionsius M, Fournier PE, Vene S, Hoel T, Hassle G, Henriksen AZ, Hellum KB, Raoult D, Myrvang B, and the Norwegian African Tick Bite Fever Study Group, 2003. African tick bite fever in travelers to rural sub-equatorial Africa. *Clin Infect Dis* 36: 1411–1417.
- Walker AR, Bouattour A, Camicas JL, Estrada-Pena A, Horak IG, Latif AA, Pegram RG, Preston PM, 2003. *Ticks of Domestic Animals in Africa; A Guide to Identification of Species*. Atalanta Houten, The Netherlands: Biosciences Reports.
- Labruna MB, Whitworth T, Horta MC, Bouyer DH, McBride JW, Pinter A, Popov V, Gennari SM, Walker DH, 2004. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of Sao Paulo, Brazil, where Brazilian spotted fever is endemic. *J Clin Microbiol* 42: 90–98.
- Regnery RL, Spruill CL, Plikaytis BD, 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J Bacteriol* 173: 1576–1589.
- Fournier PE, Jensionsius M, Laferl H, Vene S, Raoult D, 2002. Kinetics of antibody responses in *Rickettsia africanae* and *Rickettsia conorii* infections. *Clin Diagn Lab Immunol* 9: 324–328.
- Smoak BL, McClain JB, Brundage JF, Broadhurst L, Kelly DJ, Dasch GA, Miller RN, 1996. An outbreak of spotted fever rickettsioses in U.S. Army troops deployed to Botswana. *Emerg Infect Dis* 2: 217–221.
- Niebylski ML, Peacock MG, Schwan TG, 1999. Lethal effects of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). *Appl Environ Microbiol* 65: 773–778.