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Identification of *Rickettsia conorii* Infection by Polymerase Chain Reaction in a Soldier Returning from Somalia

Wheaton J. Williams, Suzana Radulovic,
Gregory A. Dasch, Jill Lindstrom, Daryl J. Kelly,
Charles N. Oster, and David H. Walker

From the Infectious Disease Service and the Dermatology Service, Walter Reed Army Medical Center, Washington, D.C.; the Department of Pathology, University of Texas Medical Branch, Galveston, Texas; and the Viral and Rickettsial Disease Program, Naval Medical Research Institute, Bethesda, Maryland

A soldier developed characteristic manifestations of boutonneuse fever shortly after leaving Somalia. Rickettsial DNA was detected in a biopsy sample of the tache noire by a polymerase chain reaction (PCR) in which primers derived from the 190-kD antigen gene of *Rickettsia rickettsii* were used. The source of this DNA was identified as *Rickettsia conorii* by restriction fragment length polymorphism (RFLP) analysis of the PCR product. *R. conorii* was also isolated from the skin biopsy specimen. The patient did not develop a significant increase in specific antibodies, as assessed by indirect fluorescent antibody testing, until several weeks after the onset of symptoms. This case demonstrates that the PCR/RFLP technique can be used for the direct identification of rickettsiae from clinical specimens. To our knowledge, this is the first confirmed case of *R. conorii* infection in Somalia.

The deployment of U.S. forces to Somalia exposed large numbers of soldiers to infectious diseases endemic to the region, including rickettsial diseases [1]. While information on rickettsial infections in Somalia is limited, these diseases have been well described in neighboring countries, and a serological survey of Somali refugees has suggested past exposure to rickettsial organisms of the spotted fever group (SFG), possibly including *Rickettsia conorii* [2]. Tick typhus due to *R. conorii* is the rickettsial infection most commonly imported by North American travelers, with most cases acquired in eastern or southern Africa [3, 4].

R. conorii is transmitted to humans by *Rhipicephalus sanguineus* and possibly by a variety of other ixodid ticks as well. Infection with this organism results in an acute illness characterized in its classic form by fever, maculopapular rash, and an eschar at the site of the tick bite (the "tache noire") [5]. The diagnosis can be confirmed by serological testing, by isolation of the organism from blood or skin, or by immunohistologic or immunocytological demonstration of the organism in skin biopsy samples or in circulating endothelial cells [6-16]. Serological testing is generally not helpful in evaluating the acutely ill patient, as most patients do not seroconvert until after day 10 of the illness [5]. Rickettsiae can be

isolated in a timely manner by shell vial centrifugation-enhanced cell culture, but few laboratories undertake this method [8]. The identification of the isolate by serological techniques is hampered by cross-reactivity among SFG rickettsiae, which is addressed by the preparation of specific murine typing sera or monoclonal antibodies [17, 18]. The polymerase chain reaction (PCR) provides a means for the rapid detection of rickettsial DNA in clinical samples [19], and restriction fragment length polymorphism (RFLP) analysis of the PCR product is useful for the identification of rickettsiae [20]. We report here a case of boutonneuse fever acquired in Somalia in which the diagnosis was confirmed by PCR/RFLP analysis as well as by isolation of *R. conorii* from the tache noire.

Case Report

A 36-year-old man spent 3 days in Somalia in late December 1992. He stayed in a tent in the U.S. Embassy compound in Mogadishu each night but traveled extensively in rural areas during the day. He always wore his military fatigues but never used insect repellent. He did not recall any tick bites or contact with animals. On his return trip to the United States, he stayed overnight at a modern hotel in Singapore and was unaware of having sustained any tick or mite bites.

About 10 days after he left Africa, he developed abrupt-onset fever, headache, neck stiffness, arthralgias, and myalgias. Three days later, he noticed a black skin lesion on his right calf. He sought medical attention in a local emergency room and was given cephalexin for a presumed upper respiratory infection; however, his symptoms were not alleviated. One week after the onset of his illness, he developed a non-

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Reprints or correspondence: Dr. Wheaton Williams, Infectious Diseases Clinic, Department of Medicine, Fitzsimons Army Medical Center, Aurora, Colorado 80045-5001.

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Figure 1. Photograph of a 2-cm eschar ("tache noire") on the right calf of a soldier who acquired *R. conorii* infection in Somalia.

productive cough. He presented to Walter Reed Army Medical Center 2 days later.

On examination, he had a temperature of 102.6°F and a faint maculopapular eruption involving the chest, abdomen, face, proximal upper extremities, and palms. Bilateral palpebral conjunctivitis was evident. A 2-cm (in diameter) erythematous lesion with a black center was noted on his right calf (figure 1). A 2-cm mobile, tender right femoral lymph node was also documented. No evidence of meningeal irritation was found. The lungs were clear.

Laboratory evaluation revealed a white blood cell (WBC) count of $9.6 \times 10^3/\mu\text{L}$, a hematocrit of 42%, a platelet count of $318,000/\mu\text{L}$, a serum aspartate aminotransferase level of 129 IU/L, and a serum alanine aminotransferase level of 391 IU/L. A lumbar puncture yielded CSF containing 25 WBCs/ μL (100% mononuclear cells), no red blood cells, 32 mg of protein/dL, and 67 mg of glucose/dL. A chest radiograph revealed an increased density in the right base consistent with an infiltrate. Punch biopsy samples of the eschar were obtained.

On the basis of epidemiological and clinical features, spotted fever rickettsiosis was diagnosed and the patient received a 5-day course of doxycycline (100 mg by mouth two times daily). He had a dramatic response to therapy and was afebrile within 24 hours.

Materials and Methods

Immunohistology. A biopsy specimen from the eschar was shipped frozen on dry ice to the Rickettsiology Laboratory, Department of Pathology, University of Texas Medical Branch, Galveston. There the specimen was thawed and divided into two portions: one for PCR and rickettsial isolation and the other for immunohistologic assay of SFG rickettsiae.

The tissue for the immunohistologic studies was fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Sections (5 μm thick) were cut and processed as previously described for immunofluorescence and immunoperoxidase demonstration of *R. conorii* [10, 21].

Serological studies. Microimmunofluorescence testing was conducted as described previously [22]. The rickettsial species that were used as antigens in the indirect fluorescent antibody (IFA) test included *R. conorii* (Malish 7 and Moroccan strains), *Rickettsia africae* (Zim F) [23], Thai tick typhus (TT-118), Israeli tick typhus (ISTT T-487), *Rickettsia typhi* (Wilmington strain), and *Rickettsia tsutsugamushi* (Karp, Gilliam, and Kato strains). Sera were tested as serial twofold dilutions from 1:16 to 1:512 against each antigen, and the results were read at 40X with a fluorescence microscope (Photomicroscope III; Zeiss, Oberkochen, Germany). Fluorescein-conjugated rabbit anti-human IgG (heavy- and light-chain-reaction) was obtained from Cappel Laboratories (Cochranville, PA). The end-point titer recorded was the highest dilution with distinct fluorescing rickettsiae.

The western blot assay was performed as previously described except that the antigens were purified by Renografin (Squibb, New Brunswick, NJ) density-gradient centrifugation [24]. Rickettsial antigens used in the immunoblots included those of *Rickettsia sibirica* (246-PP [plaque purified]), *Rickettsia slovaca* (B strain), *R. conorii* (Kenya tick typhus strain), *R. africae* (Ethiopian spotted fever strain ETH SF2500), Israeli tick typhus (ISTT T-487), *Rickettsia akari* (H5564), *Amblyomma americanum* tick isolate (OSU 85-1034), *Rickettsia bellii* (OT-2006), *R. typhi* (Wilmington strain), and *Rickettsia prowazekii* (Breinl strain). The patient's sera were allowed to react with the electrophoresed antigens at a 1:100 dilution. Antibody binding was detected with goat anti-human immunoglobulin γ - and μ -chain-specific horseradish peroxidase conjugates (CalBiochem Behring, La Jolla, CA) and 4-chloro-1-naphthol histochemical stain.

Isolation and identification of rickettsiae. A monolayer of African green monkey kidney (Vero) cells with minimal essential medium containing 1% fetal bovine serum, 2 mM L-glutamine, and antibiotics (gentamicin, 4 $\mu\text{g}/\text{mL}$; sulfamethoxazole, 6 $\mu\text{g}/\text{mL}$; trimethoprim, 0.064 $\mu\text{g}/\text{mL}$; and amphotericin B, 5 $\mu\text{g}/\text{mL}$) in a 150-cm² flask was inoculated with a homogenized sample of the biopsy specimen from the tache noire. At 7-day intervals, the cells were trypsinized for

passage, and a sample was stained by the Gimenez method [24a] for detection of the growth of organisms.

Heavily infected cells were harvested from the culture flask and stored at -80°C until used for microimmunofluorescence serotyping, PCR/RFLP analysis, and transmission electron microscopy. Murine typing antisera were prepared for *R. conorii* (Moroccan, Malish 7, and Kenyan tick typhus strains), *Rickettsia rickettsii* (Sheila Smith strain), Israeli spotted fever rickettsial isolate (A828), Astrakhan spotted fever rickettsial strain, *Rickettsia helvetica*, *R. slovaca*, *R. akari* (Kaplan strain), and JC-880 (an unnamed Pakistani SFG rickettsial species) by intravenous inoculation of rickettsiae into the tail vein of adult Swiss-Webster mice according to the method of Philip et al. [17]. Mice received a similar intravenous booster immunization 7 days later. Sera were collected 10 days after the first immunizing dose, pooled, and stored at -70°C . Typing sera and monoclonal antibodies that are species-specific for *R. conorii* (U14) and *R. rickettsii* (5C10F₃) were allowed to react with acetone-fixed antigen slides of the human Somali isolate and the homologous rickettsial strain [25].

Detection and identification of SFG rickettsial DNA in the tache noire by PCR. *Rickettsia*-specific DNA sequences in tache noire specimens, in the rickettsial isolate, and in prototype strains of SFG rickettsiae were amplified by PCR: DNA sequences from the gene of the 190-kD surface protein of *R. rickettsii* were used as oligonucleotide primers as described previously [20]. PCR was performed with use of 10 μL of the boiled tache noire suspension as a DNA template. A pair of oligonucleotide primers previously designated as Rr 190.602_p (5'-ATGGCGAATATTTCTCCA-3') and Rr 190.70_p (5'-AGTGCAGCATTCGCTCCCCCT-3') were employed. PCR amplification was carried out for 35 cycles of denaturation (20 seconds at 95°C), annealing (30 seconds at 48°C), and extension (2 minutes at 60°C), and PCR-amplified DNA was detected by rapid agarose electrophoresis of a 10- μL volume of the PCR product.

Analysis of amplified DNA by RFLP. Restriction endonuclease digestion of 10 μL of the PCR products followed standard techniques, with incubation at 37°C [26]. *Pst*I endonuclease was obtained from Promega (Madison, WI).

After the addition of dye-Ficoll loading mixture, the digested reactions were loaded on 1.5-mm-thick, 8% polyacrylamide vertical gels made by standard procedures [26]. The oligonucleotides were separated by electrophoresis at 200 V for 1 hour. The gels were then stained with ethidium bromide before illumination with an ultraviolet light source and were photographed with Polaroid type 107C film (Sigma Chemical, St. Louis).

Results

Evaluation of frozen sections of the tissue sample by immunofluorescence and immunoperoxidase failed to reveal SFG



Figure 2. Biopsy sample from an eschar on the right calf of a soldier who acquired *R. conorii* infection in Somalia. Note the necrosis of the epidermis and superficial dermis (arrow) and the perivascular lymphohistiocytic infiltrate (between the arrowheads) with endothelial cell swelling (hematoxylin and eosin, $\times 140$).

rickettsiae, although blood vessels with a perivascular lymphohistiocytic infiltrate characteristic of the host response to rickettsial infection were identified by light microscopy (figure 2).

The IFA tests confirmed the diagnosis of SFG rickettsiosis: the patient had an eightfold rise in titer of antibody to the Moroccan strain of *R. conorii* (table 1). The patient's acute-phase serum had a significant level of antibodies to the Malish 7 strain of *R. conorii*, but no significant rise in antibody titer from this level was noted in convalescent-phase serum.

Western blot immunoassays were conducted with sera obtained at all three time points (acute phase, convalescent phase, and late convalescent phase) to confirm that the low titer of antibodies detected by IFA tests were characteristic of rickettsial infections (figure 3) [7, 24]. Strong IgM and weaker IgG antibodies to the variable large and small surface protein antigens (bands >90 kD) were observed with all isolates except *R. bellii*, which lacks these antigens (figure 3, lane 8). No apparent specificity for the bands of a single species (as documented for some sera) was found. Although

Table 1. Diagnostic serology for rickettsial diseases: evaluation of a soldier who acquired *R. conorii* infection in Somalia.

Antigen*	IgM/IgG titer at indicated time (mo/ d/y)		
	Acute (1/17/93)	Con- valescent (2/11/93)	Late convalescent (3/10/93)
<i>R. conorii</i> (Malish 7 strain)	64/64	64/64	16/128
<i>R. conorii</i> (Moroccan strain)	16/16	64/32	16/128
<i>R. africae</i> (Zim F)	16/16	<16/32	16/32
TT-118	<16/<16	16/64	16/32
ISTT (T-487)	16/<16	<16/<16	16/32
<i>R. typhi</i> (Wilmington strain)	<16/<16	16/32	32/64
<i>R. tsutsugumushi</i> (Karp strain)	<16/<16	<16/<16	<16/<16
<i>R. tsutsugumushi</i> (Gilliam strain)	<16/<16	<16/<16	<16/<16
<i>R. tsutsugumushi</i> (Kato strain)	<16/<16	<16/<16	<16/<16

NOTE. Sera were screened at a 1:16 dilution by the IFA test with fluorescein isothiocyanate-labeled goat anti-human IgG and IgM. Generally, an IFA titer of ≥ 64 or a rise in titer to ≥ 64 in the appropriate clinical setting is considered diagnostic.

* TT-118 = Thai tick typhus; ISTT = Israeli tick typhus.

anti-lipopolysaccharide (LPS) reactivity (20- to 50-kD region) is common with most typhus and SFG rickettsial infections, only the LPS of *R. sibirica* (figure 3, lane 1) and the unique washboard pattern of the higher-molecular-weight LPS of *R. bellii* (33–90 kD, figure 3, lane 8) could be detected with IgM. Serum reactivity was also atypical in terms of the weak IgM reactivity and the very pronounced IgG reactivity with the 60-kD protein (GroEl homologue, HSP 60) present in each of the isolates. No significant differences in intensity or antigenic specificity were observed among the three sera tested by western blot (data not shown), despite the somewhat higher antibody titers observed by IFA testing.

Rickettsiae were detected in the second passage after 14 days in cell culture. Reaction with typing sera demonstrated that the Somali isolate was an SFG rickettsia antigenically most closely related to the Moroccan strain of *R. conorii* (table 2). Transmission electron microscopy revealed small intracellular gram-negative bacilli morphologically typical of SFG rickettsiae.

PCR amplification of DNA extracted from the rickettsial isolate with use of primers for the 190-kD gene yielded a distinct DNA product (figure 4). RFLP analysis of the PCR-amplified DNA from SFG rickettsiae encoding sequences of the 190-kD gene showed that the Somali isolate was indistinguishable from two standard strains of *R. conorii* (figure 5).

Discussion

Previous studies have demonstrated that PCR can detect rickettsial DNA in clinical samples. PCR amplification with oligonucleotide primers based on the DNA sequence of the 17-kD antigen gene from *R. rickettsii* has been used for the retrospective detection of rickettsiae in blood clots from patients with acute Rocky Mountain spotted fever and acute typhus and in serum and CSF from a seronegative patient with a fatal rickettsial infection [19, 27, 28]. The 17-kD antigen is an outer-membrane protein with characteristics of a lipoprotein, and its gene sequence is highly conserved among typhus and SFG rickettsiae. Consequently, PCR is not useful in distinguishing between typhus-group and SFG rickettsial infections if the primers used are based on conserved regions of the 17-kD antigen gene [29, 30]. RFLP analysis of the PCR product from the 17-kD antigen gene primers differentiates SFG from typhus-group rickettsiae but does not differentiate among closely related SFG rickettsiae [19, 29].

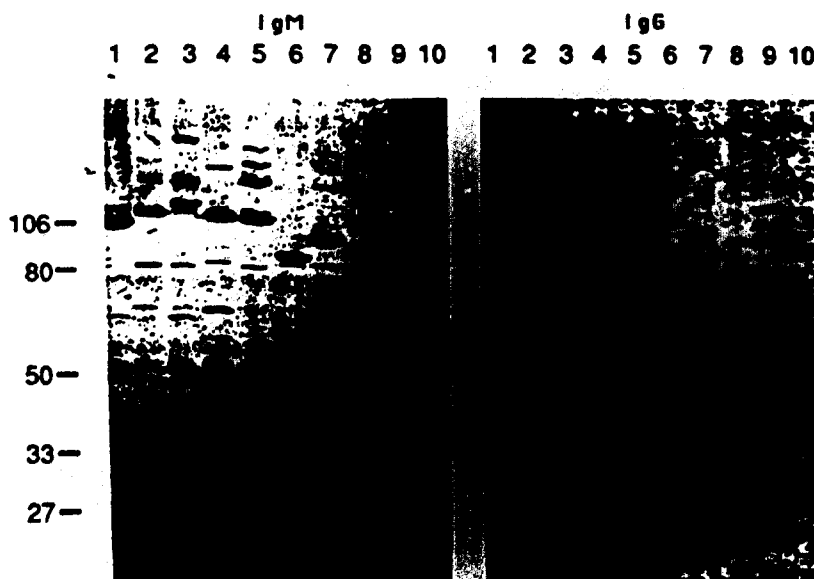


Figure 3. Western blot immunoassay reactivity of acute-phase serum diluted 1:100 with antigens from typhus and spotted fever rickettsiae. Molecular-weight markers (in kilodaltons) are shown at the left. Lane 1, *R. sibirica* (246 PP [plaque purified]); lane 2, *R. slovaca* (B); lane 3, *R. conorii* (Kenya tick typhus); lane 4, *R. africae* (ETH SF2500); lane 5, Israeli tick typhus (ISTT T-487); lane 6, *R. akari* (H5564); lane 7, *A. americanum* tick isolate (OSU 85-1034); lane 8, *R. bellii* (OT-2006); lane 9, *R. typhi* (Wilmington); and lane 10, *R. prowazekii* (Breinl).

The identification of SFG rickettsiae through PCR technology has been accomplished by RFLP analysis of PCR products from primers based on the DNA sequence of the 190-kD antigen gene of *R. rickettsii* [20]. Since the 190-kD surface protein antigen gene contains both conserved and variable sequences, it is useful for PCR/RFLP analysis [20, 31]. The PCR/RFLP technique has been used to demonstrate the genotypic variation among SFG rickettsiae and to distinguish between Israeli SFG rickettsial clinical isolates and reference strains of *R. conorii* [32]. Our case confirms that the PCR/RFLP technique can be used for the accurate direct identification of rickettsiae from clinical specimens.

This case illustrates the difficulty of confirming the diagnosis of rickettsial diseases and the ineffectiveness of many antimicrobial regimens that are applied empirically to undiagnosed illnesses of suspected infectious origin. Moreover, the pulmonary manifestations of nonproductive cough and a radiographic infiltrate demonstrate that this patient had substantial visceral involvement. *R. conorii* infection is occasionally severe or even fatal [33, 34].

This case also documents the utility of PCR as a diagnostic tool in rickettsial infections and highlights the limitations of available serological and immunohistologic tests. The patient had classic clinical manifestations of boutonneuse fever for about 10 days before receiving appropriate antibiotic therapy but did not develop a significant increase in specific antibodies—as assayed by standard serological methods—until several weeks after the onset of his symptoms. In addition, western blot immunoassays demonstrated that the patient

Table 2. Reactivity of the Somali SFG rickettsial isolate with murine typing sera and monoclonal antibodies (Mabs)

Typing serum/Mab	IFA titer with	
	Somali human isolate	Homologous rickettsial strain
Typing serum		
<i>R. conorii</i> (Moroccan strain)	2,048	1,280
<i>R. conorii</i> (Malish 7 strain)	1,024	16,384
<i>R. conorii</i> (Kenya tick typhus)	512	2,048
<i>R. conorii</i> (Sheila Smith strain)	<16	8,192
Israeli spotted fever strain (A828)	64	16,384
Astrakhan spotted fever strain	64	16,384
Pakistani spotted fever strain (JC-880)	256	8,192
<i>R. akari</i> (Kaplan strain)	<16	10,240
<i>R. sibirica</i> (B strain)	64	2,048
<i>R. helvetica</i>	<16	1,024
<i>R. typhi</i> (Wilmington strain)	<16	512
Mab		
Mab U ₁₂	8,192	8,192*
Mab SC10-E ₁	<16	16,384

* Titer vs. *R. conorii*.

* Titer vs. *R. sibirica*.



Figure 4. Ethidium bromide-stained agar gels of PCR products of the Somali SFG rickettsial isolate, with use of primers for the 190-kD SFG antigen gene and positive and negative controls. From left: lane 1, 123-bp DNA ladder molecular markers; lane 2, λ phage digested with *BstEII*; lane 3, λ phage digested with *HindIII*; lane 4, PCR-positive control (λ primers and DNA [gene Amp[®] PCR reagent kit]); lane 5, PCR product with *R. conorii* (Moroccan strain); lane 6, PCR product with *R. rickettsii* (Sheila Smith strain); and lane 7, PCR product with Somali SFG rickettsial isolate.

did not develop significant amounts of antibody to the epitopes unique to either typhus or SFG LPS, which typically are highly immunogenic in most infections with these rickettsiae [7, 24]. The immunoblot patterns were atypical of rickettsial infections in their relatively weak reactivity with LPS. With IgM, such reactivity could be discerned only as a weak, broad washboard pattern with *R. sibirica* and *R. helvetica* antigens; with IgG, it could not be discerned at all. In higher-titered sera, this reactivity is observed with all SFG antigens and both classes of antibodies. The prominent reactivity of the 90- to 200-kD serotype protein antigens is typical of rickettsial infections (in this case, strong and broad reactivity with IgM, weaker and more restricted with IgG). About 10% of sera from patients with SFG infections exhibit reactivity

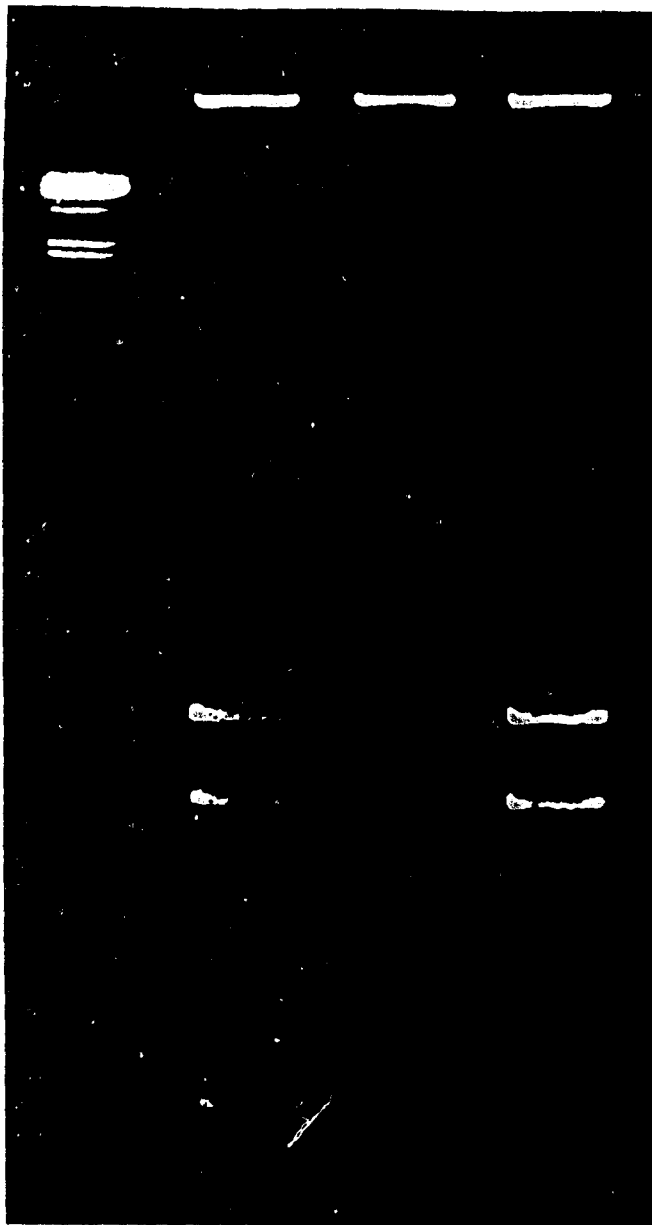


Figure 5. RFLP analysis of SFG rickettsial DNA from the tache noire and from prototype SFG rickettsiae amplified with primers for a 532-bp sequence of the 190-kD protein gene, digested with *Pst*I restriction endonuclease, separated in an 8% acrylamide gel, and stained with ethidium bromide. From left: lane 1, λ phage digested with *Hind*III; lane 2, *R. conorii* (Malish 7 strain); lane 3, *R. conorii* (Moroccan strain); and lane 4, digested PCR product obtained directly from the tache noire.

toward the conserved 60-kD heat-shock protein (in this case, prominent reactivity with IgG and weak with IgM).

Although our patient had a significant level of antibodies to *R. conorii* detected by IFA testing in his first serum sample, he did not promptly develop a substantial rise in antibody titer. In contrast, evaluation of all three sera by a novel, defined, epitope-blocking enzyme immunoassay revealed

species-specific antibodies to *R. conorii* (S. Radulovic and D. H. Walker, unpublished data). This assay offers the possibility not only of determining that the IFA reactivity was stimulated by the pathogenic rickettsial species *R. conorii* but also of making earlier serological diagnosis. It is unlikely that therapy with cephalexin affected the patient's antibody response, as β -lactam drugs are not active against rickettsiae [35]. Immunofluorescence and immunoperoxidase staining of a biopsy sample from the tache noire failed to reveal rickettsiae.

The use of PCR not only led to a diagnosis of rickettsial infection but also established the species of the infecting organism. PCR of infected skin-rash biopsy samples may also be helpful in the rapid diagnosis of boutonneuse fever in cases lacking an eschar. These cases are difficult to distinguish from other acute febrile illnesses with exanthem. An eschar often is not recognized and frequently is not present [3]. When patients have no rash, circulating endothelial cells captured by immunomagnetic beads may provide an appropriate sample for the sensitive detection of SFG rickettsial DNA [12]. Clinical samples that have been used for the diagnosis of rickettsial infection by PCR analysis include blood clot, serum, CSF, and tissue obtained at necropsy or at cutaneous biopsy. Samples may be processed immediately or stored at -70°C until examined. PCR amplification of DNA extracted from formalin-fixed tissues requires particular care for the successful detection of rickettsial material [36]. PCR is a promising diagnostic technique for rickettsial infections because it can rapidly detect small numbers of rickettsiae in easily obtained clinical samples, thus establishing a diagnosis earlier in the clinical course than is possible with serological testing. PCR should prove more sensitive than immunohistologic staining techniques because it evaluates a larger volume of tissue. The isolation of rickettsiae in cell culture is no more difficult than the isolation of cytomegalovirus—a routine procedure in many hospitals—but usually requires 72 hours. The timely treatment of SFG rickettsioses depends on clinical and epidemiological acumen and a high index of suspicion. Ideally, these specific laboratory tests will be used to confirm the diagnosis early in the course of treatment.

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