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Characterization of and Application of Monoclonal Antibodies against *Rickettsia africae*, a Newly Recognized Species of Spotted Fever Group Rickettsia

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Rickettsia africae **is a newly described species which causes African tick bite fever. Mediterranean spotted fever caused by *R. conorii* is endemic in the same regions of Africa as tick bite fever, and differentiation of the two syndromes by characterization of their etiological agents is important for epidemiological studies. *R. africae* and *R. conorii* are, however, difficult to distinguish, and therefore, our aim was to produce monoclonal antibodies to address this problem. Monoclonal antibodies were produced against *R. africae* by fusing splenocytes from BALB/C mice immunized with purified rickettsial organisms and SP2/0-Ag14 myeloma cells. A total of 355 hybridomas producing monoclonal antibodies to *R. africae* were identified by initial screening with six different antigens by microimmunofluorescence assay. A panel of 23 representative monoclonal antibodies were selected and subcloned. This panel was screened with a further 17 different spotted fever group (SFG) rickettsial reference antigens. Of these 23 monoclonal antibodies, 1 cross-reacted with only *R. parkeri*, whereas the others cross-reacted with more than two different antigens. Immunoblotting indicated that all the monoclonal antibodies were directed against the epitopes on two major high-molecular-mass heat-labile proteins, of which the molecular masses were 128 and 135 kDa, respectively. This monoclonal antibody panel was used successfully to identify *R. africae* in the blood culture of an infected patient, in infected cells within shell vials, and in infected ticks collected from Africa. Furthermore, the cross-reactivity of each SFG rickettsia with each of these 23 monoclonal antibodies was scored and was used to build a dendrogram of taxonomic relatedness between *R. africae* and the other SFG rickettsiae on the basis of Jaccard coefficients and unweighted pair group method with arithmetic mean analysis. The relatedness was generally consistent with that obtained by other methods of comparison.**

Rickettsia africae, an obligate intracellular parasite, is a new species of spotted fever group (SFG) rickettsia (36). *R. africae* is pathogenic for humans, causing African tick bite fever (34–36).

African tick bite fever has been recognized since the beginning of the century. Reports of two different kinds of rickettsioses in Africa, Mediterranean spotted fever and tick bite fever, were described as early as 1910 (43, 55), although it was not known that they were caused by different entities. In the 1930s, epidemiological variation between these two diseases was also recognized (46–48). However, following the isolation of *R. conorii* from Mediterranean spotted fever patients in Africa (15) and the demonstration of cross-immunity between Mediterranean spotted fever and African tick bite fever (23), *R. conorii* came to be regarded as the causative agent of both African rickettsioses, with African tick bite fever being attributed to *R. conorii* var. *pijperi* (24).

Recent improvements in the techniques for the isolation and characterization of rickettsiae have led to the description of several novel SFG rickettsial species (40, 52, 53). In the past few years, a number of SFG rickettsiae have been isolated and identified from distinct geographical regions, including *R. sibirica* from China (20, 63), *R. japonica* from Japan (56–58), *R. helvetica* from Switzerland (8), and *R. massiliae* from France (7, 9) and Greece (6). Kelly and colleagues recently reported the isolation of SFG rickettsial strains from a patient suffering from African tick bite fever (34) and from cattle ticks (*Amblyomma hebraeum*) in Zimbabwe (30, 33). On the basis of phenotypic and genotypic characteristics, these isolates were found to be distinct from *R. conorii* and other SFG rickettsiae (19, 29, 31, 32, 35, 52, 53). Moreover, these isolates were closely related to a previously characterized Ethiopian isolate (16) of which the pathogenicity was unknown. The name *R. africae* was proposed for this new species of SFG rickettsia (36).

The differentiation of *R. africae* and *R. conorii* led to southern Africa being recognized as the first region where two different tick-transmitted rickettsioses coexist. However, because of the intense cross-reactivity between *R. africae* and *R. conorii*, it is impossible to distinguish them serologically, and thus, complicated and time-consuming isolation and characterization methods are prerequisites for epidemiological studies. Although some molecular methods for differentiating these two rickettsiae have been developed (19, 51), their use is limited in the field because they require specialized equipment. With the aim of developing a rapid and convenient methodology for use in the clinical diagnosis, epidemiological investigation, and ecological study of these two diseases, we chose to produce monoclonal antibodies against *R. africae* Z9-Hu, the type strain which had been isolated from a patient suffering from African tick bite fever (34, 36). We characterized 23 monoclonal antibodies by screening them with 17 SFG rickettsial reference antigens and immunoblotting. We used all these monoclonal antibodies to detect *R. africae* isolated from a culture of blood from a patient and two of them to detect rickettsiae in infected shell vials and in infected ticks. The results indicate that these monoclonal antibodies are useful in distinguishing *R. africae* from *R. conorii* and other SFG rick-

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TABLE 1. Rickettsial reference antigens used in the study

Antigen[a]	Strain	Source	Geographic origin	Human disease	Collection number[b]	Reference
R. africae	Z9-Hu	Human	Zimbabwe	African tick bite fever		36
R. africae	Simon (2479)	Human	France	African tick bite fever		—[c]
R. africae	Ethiopian	*Amblyomma variegatum*	Ethiopia	African tick bite fever		16
R. akari	MK (Kaplan)	Human	New York	Rickettsial pox	ATCC VR 148	28
R. australis	Philips	Human	Australia	Queensland tick typhus		5
*Bar 29	Bar 29	*Rhipicephalus sanguineus*	Spain			10
R. bellii	369L42-1	*Dermacentor andersoni*	Ohio			44
R. conorii	Moroccan	Unknown	Morocco	Mediterranean spotted fever	ATCC VR 141	13
R. helvetica	C9P9	*Ixodes ricinus*	Switzerland		ATCC VR 1375	8
*Itt rickettsia[d]	ISTT CDC 1	Human	Israel	Israeli spotted fever		26
R. japonica	YH	Human	Japan	Oriental spotted fever	ATCC VR 1363	58
R. massiliae	Mtu 1	*Rhipicephalus turanicus*	France		ATCC VR 1376	9
*'*R. aeschlimanni*'	MC 16	*Hyalomma marginatum*	Morocco			11
R. parkeri	Maculatum 20	*Amblyomma maculatum*	Mississippi			12
R. rhipicephali	3-7-6	*Rhipicephalus sanguineus*	Mississippi			17
R. rickettsii	Sheila Smith	Human	Montana	Rocky Mountain spotted fever	ATCC VR 149	12
R. sibirica	246	*Dermacentor nuttali*	Former USSR	North Asian tick typhus	ATCC VR 151	13
*'*R. slovaca*'	13-B	*Dermacentor marginatus*	Slovakia			60
R. typhi	Wilmington	Human	North Carolina	Murine typhus	ATCC VR 144	41
Coxiella burnetii	Nine Mile Q	*Dermacentor andersoni*	Montana	Q fever	ATCC VR 616	18

[a] The unrecognized species are marked with asterisks.
[b] ATCC, American Type Culture Collection.
[c] —, novel isolate in our laboratory.
[d] Itt rickettsia, Israeli tick typhus rickettsia.

ettsiae. Furthermore, these monoclonal antibodies show potential for use in studying the antigenic specificity of *R. africae* and other SFG rickettsiae, followed by analyzing antigenic relationships between *R. africae* and other SFG rickettsiae on the basis of monoclonal antibody cross-reactivities.

MATERIALS AND METHODS

Rickettsial antigens. The sources of all strains used in this study are presented in Table 1. *R. africae* Ethiopian strain, *R. australis*, Israeli tick typhus rickettsia, *R. rhipicephali*, and "*R. slovaca*" were kindly provided by G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.). *R. bellii*, *R. japonica*, and *R. parkeri* were kindly provided by D. H. Walker (University of Texas, Galveston). *R. helvetica* was kindly supplied by W. Burgdorfer (Rocky Mountain Laboratory, Hamilton, Mont.). *R. sibirica* was obtained from Gamaleya Research Institute (Moscow, Russia). *R. africae* Simon was recently isolated in our laboratory from a patient suffering from African tick bite fever (14), and strain MC 16 was from a *Hyalomma marginatum* tick from Morocco (11). The provisional name "*R. aeschlimanni*" has been proposed for this strain (11).

SFG rickettsial strains and *R. typhi* were cultivated in L929 cell monolayers (ATCC CCL 1 NCTC clone 929; American Type Culture Collection, Rockville, Md.) in 175-cm^2 tissue culture flasks (Nunc InterMed, Roskilde, Denmark) supplemented with Earle's minimum essential medium (MEM; Eurobio, Les Ulis, France), 4% fetal bovine serum (FBS; Eurobio), and 2 mM L-glutamine (Gibco BRL, Life Technologies Ltd., Paisley, Scotland). Cultures of the SFG rickettsiae were incubated at 32°C, whereas the *R. typhi* culture was incubated at 35°C. *Coxiella burnetii* was grown on L929 cell monolayers supplemented with MEM in the presence of 5% FBS and 2 mM L-glutamine at 35°C. The infection of the cells was monitored by Gimenez staining (25). When the cells were heavily infected, they were harvested and stored at −80°C. These unpurified antigens were used to screen monoclonal antibodies; however, further treatment was required prior to their use in immunization or for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Harvested antigens were first sonicated to disrupt the infected cells, and then bacteria were separated by centrifugation, first in 25% sucrose in K36 solution (16.5 mM KH_2PO_4, 33.5 mM K_2HPO_4, 100 mM KCl, 15.5 mM NaCl) at 5,000 × *g* for 30 min and then in 28 to 45% Renografin (Radioselectan; Schering, Lys-Lez-Lannoy, France) density gradient at 120,000 × *g* for 1 h (64). The bands containing purified organisms were collected and washed twice with K36 solution at 13,500 × *g* for 15 min each time. The pellets were resuspended with distilled water, divided into aliquots, and stored at −80°C.

Production of monoclonal antibodies. Six-week-old female BALB/C mice were immunized three times intraperitoneally with 2×10^4 purified *R. africae* suspended in 0.5 ml of phosphate-buffered saline (PBS; pH 7.3; GibcoBRL) at 7-day intervals. One week after the final immunization, the mice were boosted intravenously with 4×10^3 purified organisms in 0.1 ml of PBS. Seventy-two hours later, the mice were sacrificed and their spleens were recovered, placed in RPMI 1640 medium (Gibco BRL), and then washed twice by centrifugation at 400 × *g* for 10 min. The splenocytes within this suspension were then fused with SP2/0-Ag14 myeloma cells as described previously (27). The fusion cells were suspended with hybridoma medium (Seromed, Berlin, Germany) containing 20% FBS (GibcoBRL) and 1× hypoxanthine-aminopterin-thymidine medium supplement (Sigma Chemical Co., St. Louis, Mo.) and were then distributed into 96-well microdilution plates (Nunc) at 100 μl (10^4 cells) per well; on the day before fusion each well was supplemented with 100 μl of normal splenocytes to support the hybridoma cells. The plates were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. Six days after fusion, 100 μl of the supernatant from each well was discarded and replaced with 100 μl of fresh hybridoma medium. Fourteen days after fusion, the supernatants from viable, replicating hybridoma clones were collected and screened for antibodies to *R. africae* by microimmunofluorescence (micro-IF) assay. Positive hybridoma clones were then spread and frozen as described previously (27). Representative hybridomas showing different cross-reactivities in the first screening were selected and subcloned two to three times by limiting dilution (27). The specificities of the monoclonal antibodies produced by the hybridoma clones were determined by immunoblotting. The immunoglobulin class and subclass of monoclonal antibodies were determined with an ImmunoType Mouse Monoclonal Antibody Isotyping Kit (stock no. ISO-1; Sigma). Ascitic fluids were produced by incubating 3×10^6 hybridoma cells suspended in 0.5 ml of PBS into mice 1 week after an intraperitoneal injection of 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma) (27).

Micro-IF assay. Two antigen sets were used to screen the hybridoma clones. An antigen set containing 6 different group and species antigens (*R. africae* Z9-Hu, *R. australis*, *R. conorii*, *R. rickettsii*, *R. typhi*, and *C. burnetii*) was used for the first screening, whereas an antigen set containing 17 different SFG rickettsial antigens (*R. africae* [Z9-Hu and Ethiopian strains], *R. akari*, *R. australis*, Bar 29, *R. bellii*, *R. conorii*, *R. helvetica*, Isreali tick typhus rickettsia, *R. japonica*, *R. massiliae*, MC 16 strain, *R. parkeri*, *R. rhipicephali*, *R. rickettsii*, *R. sibirica*, and "*R. slovaca*") was used for the second screening.

Antigens were applied to 24-well microscope slides with a pen nib. After air-drying, the antigens were fixed in acetone for 20 min at room temperature. The slides were either used immediately or hermetically sealed and were stored at −20°C until required. The micro-IF assay procedure of Philip et al. (45) was modified for screening of hybridoma clones. Briefly, the wells were overlaid with 15 μl of supernatant from hybridoma clones, and then the plates were incubated in a humidified chamber at 37°C for 30 min. After three 3-min washes in PBS, the slides were air-dried and then overlaid with fluorescein (dichlorotriazinyl amino fluorescein [DTAF])-conjugated goat anti-mouse IgG+IgM (heavy and light chains [AffiniPure]; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted 1:200 in PBS containing 0.2% Evans blue (BioMérieux, Marcy l'Etoile, France). The slides were incubated and washed as described above. Dried slides were mounted with Fluoprep (BioMérieux) and examined with a Zeiss epifluorescent microscope (Axioskop 20; Carl Zeiss, Göttingen, Germany)

TABLE 2. Reactivities of monoclonal antibody panel to *R. africae* Z9-Hu with different rickettsial reference antigens

Hybridoma	Immunoglobulin class and subclass	Specificity (kDa)	Reactivity with the antigen of[a]:																	
			Afr	Eth	Aka	Aus	Bar	Bel	Con	Hel	Itt	Jpn	Mas	MC	Par	Rhi	Ric	Sib	Slo	Typ	Cox
AF1-B9	IgG1	135	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−
AF1-D12	IgG1	135	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
AF1-F11	IgG2a	128	+	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−
AF2-D4	IgG1	128	+	+	−	−	−	−	+	−	+	+	−	−	+	+	+	+	+	−	−
AF2-D6	IgM	135	+	+	−	−	−	−	−	+	+	+	−	−	+	−	+	+	+	−	−
AF2-D7	IgG2b	128	+	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−
AF3-A2	IgG2b	128	+	+	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−
AF4-C8	IgG1	135	+	+	−	−	−	−	+	−	+	−	−	−	+	−	−	−	+	−	−
AF4-G8	IgG1	128	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−
AF5-D8	IgG2a	128	+	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	+	−	−
AF5-G8	IgG3	128	+	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−
AF5-E12	IgG2b	128	+	+	−	−	−	−	−	−	−	+	−	−	+	−	−	+	+	−	−
AF6-C1	IgG3	128	+	+	−	−	−	−	−	−	−	−	+	−	+	−	−	+	+	−	−
AF6-D1	IgG2a	128	+	+	−	−	+	−	+	−	+	+	+	−	+	+	+	+	+	−	−
AF6-D6	IgG1	128	+	+	+	+	+	−	+	−	+	+	+	−	+	+	+	+	+	−	−
AF6-B10	IgG1	128	+	+	−	−	−	−	+	−	+	+	−	−	+	−	−	+	+	−	−
AF6-E10	IgG1	135	+	+	−	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−
AF7-H9	IgG1	128	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−
AF7-F10	IgG1	135	+	+	−	−	−	−	+	−	+	+	−	−	+	−	+	+	+	−	−
AF8-F3	IgG3	128	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−
AF8-G8	IgG1	128	+	+	−	−	−	−	+	−	+	+	−	−	+	−	+	+	+	−	−
AF8-D11	IgG1	128	+	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−
AF8-D12	IgG2a	128	+	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	−
Total no.[b]	23	23	23	21	1	1	2	0	8	1	14	9	3	0	21	5	7	15	16	0	0

[a] +, reactivity; −, no reactivity. The antigens were *R. africae* Z9-Hu strain (Afr), *R. africae* Ethiopian strain (Eth), *R. akari* (Aka), *R. australis* (Aus), Bar 29 (Bar), *R. belli* (Bel), *R. conorii* (Con), *R. helvetica* (Hel), Israeli tick typhus rickettsia (Itt), *R. japonica* (Jpn), *R. massiliae* (Mas), MC 16 strain (MC), *R. parkeri* (Par), *R. rhipicephali* (Rhi), *R. rickettsii* (Ric), *R. sibirica* (Sib), "*R. slovaca*" (Slo), *R. typhi* (Typ), and *C. burnetii* (Cox).

[b] The total number of monoclonal antibodies showing cross-reactivity with screening antigens.

at a ×400 magnification. In each screening, pooled sera from mice used to produce immunized splenocytes were used as a positive control and pooled sera from healthy mice were used as a negative control.

SDS-PAGE and immunoblotting. SDS-PAGE was performed by the method of Laemmli (37). The rickettsial antigens were electrophoretically separated in a 7.5% resolving gel and a 3.0% stacking gel in the presence of 0.5% SDS at a constant current of 8 mA per gel for 3 h in an electrophoretic cell (Mini Protein II; Bio-Rad, Richmond, Calif.) held in an ice bath and with prechilled running buffer (1.25 M Tris, 1.92 M glycine, 1% SDS). Prestained SDS-PAGE standards (High-Range; Bio-Rad) were included in each run.

The separated polypeptides were transferred onto a 0.45-μm-pore-size nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad) by electrophoresis at 50 V for 1.5 h in an electrophoretic transfer cell (Mini Trans-Blot; Bio-Rad) held in an ice bath and by using prechilled transfer buffer (2.5 mM tris base, 192 mM glycine, 20% methanol). After transfer, the nitrocellulose membrane was incubated overnight with 5% nonfat powdered milk in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5], 250 mM NaCl, 0.01% [wt/vol] merthiolate) at room temperature. The membranes were washed three times for 10 min each time with TBS, air-dried, and then cut into strips. The strips were incubated in a different hybridoma supernatant or ascitic fluid diluted either 1:8 or 1:1,000, respectively, for 2 h at room temperature on a rocker. After three washes as described above, the strips were incubated for 1 h at room temperature with peroxidase-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (heavy and light chains [AffiniPure]; Jackson ImmunoResearch) diluted 1:1,000 in TBS containing 3% nonfat powdered milk. The strips were washed again, and the bound peroxidase was detected by using the substrate mixture of 0.015% 4-chloro-1-naphthol (Sigma) in 16.7% methanol in TBS containing 0.015% hydrogen peroxide. When bands were clearly visible, the reaction was stopped by repeat washes with distilled water. Controls were used as described for the micro-IF assay.

Detection of rickettsiae in shell vials. Shell vials with cover slips (7 ml; Bibby Sterilin Ltd., Stone, England) were supplemented with 1 ml of L929 cell passage suspension in MEM as described above and were incubated at 37°C in a 5% CO_2 atmosphere. Two days later, these cell monolayers were infected with 10^4 organisms by centrifugation at 700 × *g* for 30 min at room temperature. After discarding the supernatant, 1 ml of fresh MEM was added to each shell vial, and the vials were incubated for 3 days at 37°C in a 5% CO_2 atmosphere. Infecting bacteria were detected in cells by the micro-IF assay as described above. Three strains of *R. africae* (Z9-Hu strain, Simon strain, and Ethiopian strain) and one strain of *R. conorii* (Moroccan strain) were used to infect cells. Two ascitic fluids, which were derived from hybridomas AF1-D12 and AF8-F3, were diluted 1:1,000 in PBS and were used to detect the rickettsiae.

Detection of rickettsiae in tick preparations. Seven *A. hebraeum* ticks, which were collected from Zimbabwe in 1992 and which were subsequently stored at −80°C, were thawed, and their salivary glands were dissected. These glands were smeared onto microscope slides. SFG rickettsiae were detected in these smears by the micro-IF assay described above. The antigen of *R. africae* Z9-Hu was used as a positive control, and normal L929 cells were used as a negative control. The ascitic fluids derived from hybridomas AF1-D12 and AF8-F3 were used at 1:1,000 dilutions for the detection of rickettsiae. Attempts were also made to detect and identify infecting SFG rickettsiae in these ticks by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified citrate synthase and gene fragments encoding the 190-kDa protein (PCR-RFLP) by previously described protocols (19, 51).

Numerical taxonomic analysis. The reactivities of the SFG rickettsial antigens with this panel of 23 monoclonal antibodies was determined by the micro-IF assay. A positive cross-reactivity of each rickettsial antigen with the corresponding monoclonal antibody was given a score of 1. Conversely, a negative reaction was given a score of 0. These scores were then used to construct a dendrogram (54) by the unweighted pair group method with arithmetic mean (UPGMA) available in the PHYLIP software package (21). The Jaccard coefficients (S_J) derived from matrix analysis were used as measures of similarity of antigenicity between *R. africae* and the other SFG rickettsiae.

RESULTS

Production of monoclonal antibody. Eight 96-well microdilution plates were used to grow hybridoma clones. Fourteen days after fusion, viable clones were observed in 483 wells, of which 355 were found to produce monoclonal antibodies against *R. africae* Z9-Hu on initial screening. The monoclonal antibodies derived from these hybridoma cells showed different cross-reactivities with the six antigens in the screening panel.

Twenty-three representative hybridomas designated by the prefix AF were selected from the 355 hybridomas described above on the basis of their reactivities with the six antigens and were screened against the second larger panel of rickettsiae (Table 1). The reactivity of each hybridoma is presented in

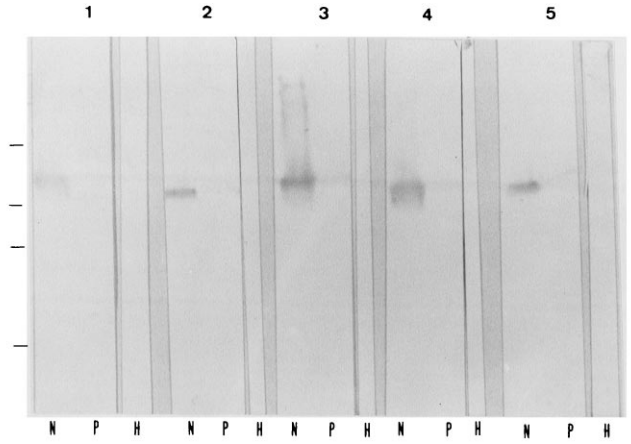

FIG. 1. Immunoblotting of *R. africae* Z9-Hu proteins treated in different ways with representative monoclonal antibodies. N, native proteins without treatment; P, antigens treated with proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at 28 U/ml at 37°C for 1.5 h; H, antigens heated at 100°C for 5 min. Group 1, AF1-D12; group 2, AF5-D8; group 3, AF6-E10; group 4, AF8-F3; group 5, AF8-D12. Molecular mass markers of 205, 116.5, 80, and 49.5 kDa (from top to bottom, respectively) were loaded on the left.

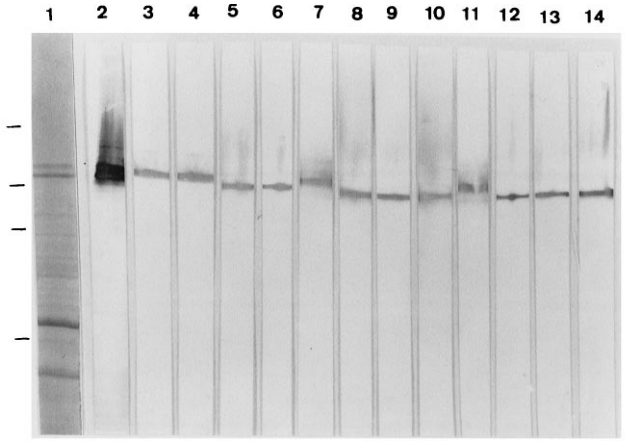

FIG. 2. Coomassie brilliant blue-stained SDS-PAGE profiles of *R. africae* Z9-Hu native proteins (lane 1) and immunoblotting with mouse polyclonal antisera (lane 2) and representative monoclonal antibodies (lane 3, AF1-B9; lane 4, AF1-D12; lane 5, AF1-F11; lane 6, AF3-A2; lane 7, AF4-C8; lane 8, AF4-G8; lane 9, AF5-E12; lane 10, AF6-D6; lane 11, AF6-E10; lane 12, AF7-H9; lane 13, AF8-F3; lane 14, AF8-D11). Molecular mass markers of 205, 116.5, 80, and 49.5 kDa (from top to bottom, respectively) were loaded on the left.

Table 2. In addition, all 23 monoclonal antibodies also reacted with the Simon strain of *R. africae* (data not shown).

Monoclonal antibody AF1-D12 cross-reacted only with *R. parkeri*, and monoclonal antibody AF1-B9 cross-reacted only with *R. parkeri* and *R. sibirica*. Other monoclonal antibodies showed cross-reactivities with more than three SFG rickettsial antigens, particularly monoclonal antibody AF6-D6, which cross-reacted with almost all SFG rickettsial reference antigens apart from *R. bellii*, *R. helvetica*, and strain MC 16 (Table 2).

Hybridomas AF1-D12, AF4-C8, AF6-D6, and AF8-F3, which exhibited different specificities for *R. africae* and the other SFG rickettsiae (Table 2), were selected to produce ascitic fluids, the homologous antibody titers of which were 1:40,960, 1:163,840, 1:40,960, and 1:81,920, respectively.

The immunoglobulin class and subclass of each of the 23 monoclonal antibodies are presented in Table 2.

SDS-PAGE and immunoblotting. The protein profile of *R. africae* could be divided into three major groups of bands (Fig. 1, lane 1): (i) high-molecular-mass bands at more than 120 kDa, (ii) middle-molecular-mass bands at 40 to 70 kDa, and (iii) low-molecular-mass bands at less than 30 kDa (lipopolysaccharide [LPS]-like bands). The mouse polyclonal antisera reacted strongly with the proteins in the high-molecular-mass range, particularly two bands of 128 and 135 kDa (Fig. 1, lane 2), although slight reactivity with proteins and LPS-like antigens in the low-molecular-mass range also occurred.

Immunoblotting demonstrated that all 23 monoclonal antibodies were directed against epitopes of one of the two previously identified high-molecular-mass proteins (Fig. 2). Of 23 monoclonal antibodies, 17 showed reactivity with the 128-kDa protein band and 6 showed reactivity with the 135-kDa protein band (Table 2). No monoclonal antibody reacted with low-molecular-mass proteins and LPS-like antigens. After proteinase K or heat treatment of antigens prior to SDS-PAGE, no bands were detected (Fig. 1).

Detection of rickettsiae in shell vials and tick preparations. Infecting rickettsiae were detected in 3-day-old cultures by the micro-IF assay incorporating monoclonal antibodies AF1-D12 and AF8-F3 (Fig. 3). The cells infected with the Z9-Hu and Simon strains of *R. africae* could be detected with both monoclonal antibodies, whereas the cells infected with the Ethiopian strain of *R. africae* could be detected only with monoclonal antibody AF8-F3. Infecting *R. conorii* strains were not detected with these two monoclonal antibodies.

Of the seven smears of salivary glands from different *A. hebraeum* ticks, six were found to be infected with SFG-like organisms by the micro-IF assay incorporating monoclonal antibody AF1-D12, whereas all seven samples were found to be infected by the micro-IF assay incorporating AF8-F3 (Fig. 4). PCR-RFLP analysis identified *R. africae* in five of these seven ticks (Table 3).

Numerical taxonomy. A dendrogram based on S_J coefficients and UPGMA analysis is presented in Fig. 5. The *R. africae* strains formed part of a cluster which also included *R. parkeri*, "*R. slovaca*," Israeli tick typhus rickettsia, *R. japonica*, *R. sibirica*, *R. rickettsii*, and *R. conorii* (Fig. 5, cluster I). Of the SFG rickettsiae listed above, *R. parkeri* and "*R. slovaca*" shared the most cross-reactivity with *R. africae*. Bar 29, *R. massiliae*, and *R. rhipicephali* showed less cross-reactivity with *R. africae* (Fig. 5, cluster II). *R. akari*, *R. australis*, and *R. helvetica* reacted with only one monoclonal antibody (Table 2), suggesting that

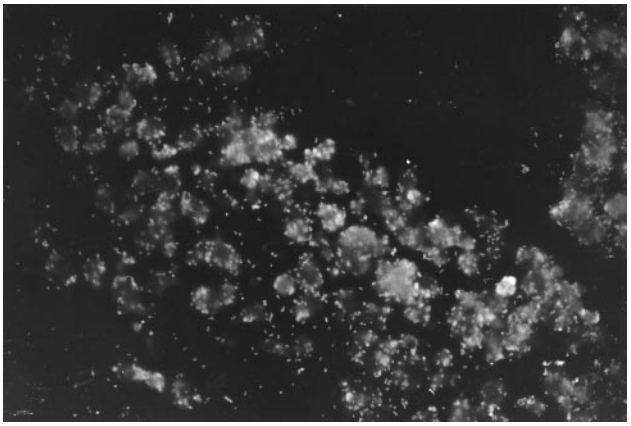

FIG. 3. Fluorescent-antibody-stained organisms of *R. africae* on a coverslip in a shell vial detected by using monoclonal antibody AF8-F3 at a dilution of 1:1,000.

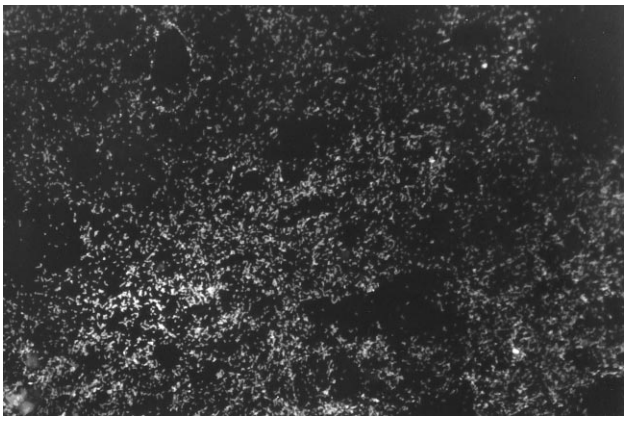

FIG. 4. Fluorescent-antibody-stained SFG-like organisms in salivary gland smear prepared from *A. hebraeum* ticks collected in Zimbabwe. Ascitic fluid AF8-F3 at a dilution of 1:1,000 was used as the antibody.

their major immunoreactive proteins are markedly different from those of *R. africae*. *R. bellii* and MC 16 did not cross-react with any of the monoclonal antibodies.

DISCUSSION

R. africae has now been identified as the second etiological agent of rickettsiosis in Africa in regions where *R. conorii* has been recognized as the causative agent of African rickettsioses for almost a century. Recently, *R. africae* has been isolated successfully from *A. hebraeum* ticks collected from wild and domestic animals (in particular, cattle) (30) and from one patient suffering from African tick bite fever (34). Very recently, four travel-associated cases of spotless rickettsioses characterized by fever, multiple tache noire, and no rash have also been demonstrated to be due to *R. africae* (14).

Phenotypic studies of *R. africae* isolates have been based on serological typing with mouse polyclonal antisera (29, 35) or monoclonal antibodies raised against *R. conorii* (32) and on SDS-PAGE and immunoblotting (31, 35). Genotypic characterization of *R. africae* isolates has used PCR-RFLP analysis (19, 35), macrorestriction analysis by pulsed-field gel electrophoresis (52), and comparison of 16S rRNA gene sequences (53). Although all these approaches have distinguished *R. africae* from *R. conorii* and other SFG rickettsiae, the antigenic differences between these species remain uncertain.

Monoclonal antibodies have previously been produced against several of the pathogenic SFG rickettsiae, including *R. akari* (42), *R. conorii* (22, 39, 61), *R. japonica* (59), *R. rickettsii* (1, 4, 38, 39), and *R. sibirica* (39), for the study of the protective antigens (39), antigenic diversity (62), or virulence (2, 3) of SFG rickettsiae. Some monoclonal antibodies have also shown potential for use in the development of vaccines (4, 39). Species-specific monoclonal antibodies have been used to determine the etiologic agent of SFG rickettsioses in large-scale surveys (49).

TABLE 3. Detection of rickettsiae in tick preparations

Detection method[a]	Result for tick no.:						
	1	2	3	4	5	6	7
MAb AF1-D12	−	+	+	+	+	+	+
MAb AF8-F3	+	+	+	+	+	+	+
PCR-RFLP analysis	−	+	+	+	+	−	+

[a] The monoclonal antibodies (MAbs) were used at a dilution of 1:1,000 with PBS in the micro-IF assay.

Our production methodology yielded a high percentage of hybridoma clones (73.5%) secreting monoclonal antibodies to *R. africae*. On primary screening, these monoclonal antibodies showed different degrees of cross-reactivity with *R. africae*, *R. australis*, *R. conorii*, and *R. rickettsii*. Analyses of these cross-reactivities suggested that *R. africae* shared higher antigenic similarity with *R. conorii* (82.0%) and *R. rickettsii* (63.4%) than with *R. australis* (6.8%), demonstrating that *R. australis* is antigenically less closely related to the other pathogenic SFG rickettsiae. This finding corresponds to those of previous studies of the phenotypic and genotypic characteristics of *R. australis* (7, 19, 49, 52, 53).

Within the SFG rickettsiae, variation among immunoreactive high-molecular-mass proteins is well documented, and these differences have been used to distinguish different species (3, 7). PCR-RFLP differentiation schemes have also been designed to exploit variations in the genes encoding the two major high-molecular-mass proteins of 190 kDa (rOmpA) and 120 kDa (rOmpB) (19, 51). Most monoclonal antibodies raised against intact SFG rickettsiae are reactive with epitopes on these immunoreactive high-molecular-mass proteins (3, 22, 39, 59), and all 23 monoclonal antibodies used in our study were also shown to be reactive, reacting with 128- and 135 kDa proteins, which were shown to be the principal immunogens of *R. africae* by immunoblotting with mouse polyclonal antisera. These two proteins were also shown to be heat labile. The investigation of the specificity of this monoclonal antibody panel showed that monoclonal antibodies to the 135-kDa protein belonged to the immunoglobulin M (IgM) class and the IgG1 subclass, while all monoclonal antibodies to the 128-kDa protein belonged to the subclasses of IgG.

That these 23 monoclonal antibodies showed high cross-reactivities with the Ethiopian strain suggests that it is a strain of *R. africae*. However, because two monoclonal antibodies (AF1-B9 and AF1-D12) did not cross-react with the Ethiopian strain, antigenic diversity among *R. africae* isolates clearly exists. Antigenic diversity has also been reported among *R. conorii* strains following analyses of their reactivities with a panel of 12 monoclonal antibodies (62). Most of our monoclonal antibodies also reacted with *R. parkeri*, reaffirming the fact that *R. parkeri* is a close antigenic relative of *R. africae*, as demonstrated previously (19, 52, 53).

R. africae infection in cells within shell vials was detected with the monoclonal antibodies. Monoclonal antibodies AF1-D12 and AF8-F3 were able to detect *R. africae* rather than *R. conorii* infection in cells, with AF8-F3 exhibiting strong reactivity with all isolates of *R. africae*. Both monoclonal antibodies show potential for use in diagnostic investigations in Africa. Moreover, *R. africae* was also detected within tick preparations with the same monoclonal antibodies. Although all ticks were found to be infected with SFG-like rickettsiae by AF8-F3, only six of them were positive with AF1-D12, and *R. africae* infection could be confirmed in only five of the ticks by PCR-RFLP analysis. These different results could be attributed to (i) variation in the sensitivities of the two monoclonal antibodies due to different levels of expression of epitope in vivo, (ii) antigenic diversity among *R. africae* strains, or (iii) the presence of amplification inhibitors. Although these results can only be considered preliminary and the differences discussed above need to be investigated further, the use of these monoclonal antibodies offers a real alternative to the molecular biology-based methods used in large surveys of infections of ticks. Although cross-reaction with *R. parkeri* was observed for both monoclonal antibodies, this species exists exclusively in the United

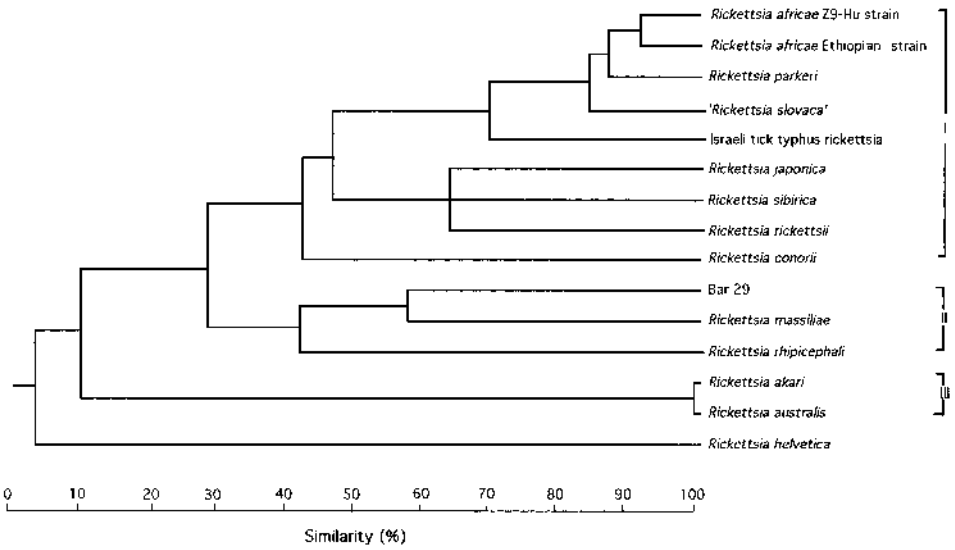

FIG. 5. Dendrogram of 17 SFG rickettsiae inferring similarities obtained by UPGMA on the basis of different cross-reactivities with 23 representative monoclonal antibodies to *R. africae* Z9-Hu.

States, and thus will not be encountered during field work in Africa.

The 23 monoclonal antibodies, which all showed different cross-reactivities with the SFG rickettsiae, were used to study the antigenic relationships between *R. africae* and other SFG rickettsiae. The dendrogram based on the cross-reactivities of SFG rickettsiae with monoclonal antibodies appears to correspond with previous taxonomic assessments of *R. africae* based on pulsed-field gel electrophoresis (52) and 16S rRNA gene sequence analysis (53).

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REFERENCES

1. **Anacker, R. L., R. H. List, R. E. Mann, S. F. Hayes, and L. A. Thomas.** 1985. Characterization of monoclonal antibodies protecting mice against *Rickettsia rickettsii*. J. Infect. Dis. **151:**1052–1060.
2. **Anacker, R. L., R. H. List, R. E. Mann, and D. L. Wiedbraunk.** 1986. Antigenic heterogeneity in high- and low-virulence strains of *Rickettsia rickettsii* revealed by monoclonal antibodies. Infect. Immun. **51:**653–660.
3. **Anacker, R. L., R. E. Mann, and C. Gonzales.** 1987. Reactivity of monoclonal antibodies to *Rickettsia rickettsii* with spotted fever and typhus group rickettsiae. J. Clin. Microbiol. **25:**167–171.
4. **Anacker, R. L., G. A. McDonald, R. H. List, and R. E. Mann.** 1987. Neutralizing activity of monoclonal antibodies to heat-sensitive and heat-resistant epitopes of *Rickettsia rickettsii* surface proteins. Infect. Immun. **55:**825–827.
5. **Andrew, R., J. M. Bonnin, and S. Williams.** 1946. Tick typhus in north Queensland. Med. J. Aust. **2:**253.
6. **Babalis, T., Y. Tselentis, V. Roux, A. Psaroulaki, and D. Raoult.** 1994. Isolation and identification of a rickettsial strain related to *Rickettsia massiliae* in Greek ticks. Am. J. Trop. Med. Hyg. **50:**365–372.
7. **Beati, L., J. P. Finidori, B. Gilot, and D. Raoult.** 1992. Comparison of serologic typing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein analysis, and genetic restriction fragment length polymorphism analysis for identification of rickettsiae: characterization of two new rickettsial strains. J. Clin. Microbiol. **30:**1922–1930.
8. **Beati, L., O. Peter, W. Burgdorfer, A. Aeschlimann, and D. Raoult.** 1993. Confirmation that *Rickettsia helvetica* sp. nov. is a distinct species of the spotted fever group of rickettsiae. Int. J. Syst. Bacteriol. **43:**521–526.
9. **Beati, L., and D. Raoult.** 1993. *Rickettsia massiliae* sp. nov.: a new spotted fever group rickettsia. Int. J. Syst. Bacteriol. **43:**839–840.
10. **Beati, L., V. Roux, A. Ortuno, J. Castella, F. Segura Porta, and D. Raoult.** 1996. Phenotypic and genotypic characterization of spotted fever group rickettsiae isolated from Catalan *Rhipicephalus sanguineus* ticks. J. Clin. Microbiol. **34:**2688–2694.
11. **Beati, L., and D. Raoult.** *Rickettsia aeschlimanni* sp. nov.: a new spotted fever group rickettsia associated with *Hyalomma marginatum* ticks. Submitted for publication.
12. **Bell, E. J., and E. G. Pickens.** 1953. A toxic substance associated with the rickettsias of the SFG. J. Immunol. **70:**461–472.
13. **Bell, E. J., and H. G. Stoenner.** 1960. Immunologic relationships among the spotted fever group of rickettsias determined by toxin neutralisation tests in mice with convalescent animal serums. J. Immunol. **84:**171–182.
14. **Brouqui, P., J. R. Harle, S. Badiaga, J. Delmont, P. J. Weiller, and D. Raoult.** African tick bite fever: an imported spotless rickettsiosis. Submitted for publication.
15. **Brumpt, E.** 1932. Longévité de virus de la fièvre boutonneuse (*Rickettsia conorii*, n. spp.) chez la tique, *Rhopicephalus sanguineus*. C. R. Soc. Biol. **110:**1199–1209.
16. **Burgdorfer, W., R. A. Ormsbee, M. L. Schmidt, and H. Hoogstraal.** 1973. A search for the epidemic typhus agent in Ethiopian ticks. Bull. W. H. O. **48:**563–569.
17. **Burgdorfer, W., D. J. Sexton, R. K. Gerloff, R. L. Anacker, R. N. Philip, and L. A. Thomas.** 1975. *Rhipicephalus sanguineus*: vector of a new spotted fever group rickettsia in the United States. Infect. Immun. **12:**205–210.
18. **Davis, G. E., and H. R. Cox.** 1938. A filter passing infections agent isolated from ticks. 1. Isolation from *Dermacentor andersoni*, reactions in animals and filtrations experiments. Public Health Rep. **53:**2259–2267.
19. **Eremeeva, M., X. J. Yu, and D. Raoult.** 1994. Differentiation among spotted fever group rickettsiae species by analysis of restriction fragment length polymorphism of PCR-amplified DNA. J. Clin. Microbiol. **32:**803–810.
20. **Fan, M. Y., X. J. Yu, and D. H. Walker.** 1988. Antigenic analysis of Chinese strains of spotted fever group rickettsiae by protein immunoblotting. Am. J. Trop. Med. Hyg. **39:**497–501.
21. **Felsenstein, J.** 1982. Numerical methods for inferring evolutionary trees. Q. Rev. Biol. **57:**379–404.
22. **Feng, H. M., D. H. Walker, and J. G. Wang.** 1987. Analysis of T-cell-dependent and -independent antigens of *Rickettsia conorii* with monoclonal antibodies. Infect. Immun. **55:**7–15.
23. **Gear, J. H. S., and B. de Meillon.** 1941. The hereditary transmission of the rickettsia of tick-bite fever through the common dog tick *Haemaphysalis leachi*. S. Afr. Med. J. **15:**389–395.
24. **Gear, J. H. S.** 1988. Other spotted fever group rickettsioses: clinical signs, symptoms, and pathology, p. 101–115. *In* D. H. Walker (ed.), Biology of rickettsial diseases, vol. 1. CRC Press, Inc., Boca Raton, Fla.
25. **Gimenez, D. F.** 1964. Staining rickettsiae in yolk sac culture. Stain Technol. **39:**135–140.
26. **Goldwasser, R. A., Y. Steiman, W. Klingberg, T. A. Swartz, and M. A. Klingberg.** 1974. The isolation of strains of rickettsiae of the spotted fever group in Israel and their differentiation from other members of the group by immunofluorescence methods. Scand. J. Infect. Dis. **6:**53–62.
27. **Harlow, E., and D. Lane.** 1988. Monoclonal antibodies, & growing hybridomas, p. 139–282. *In* E. Harlow and D. Lane (ed.), Antibodies: a laboratory

manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Huebner, R. J., P. Stamps, and C. Armstrong.** 1946. Rickettsialpox—a newly recognized rickettsial disease. Public Health Rep. **61:**1605.
29. **Kelly, P. J., and P. R. Mason.** 1990. Serological typing of spotted fever group rickelttsia isolates from Zimbabwe. J. Clin. Microbiol. **28:**2302–2304.
30. **Kelly, P. J., D. Raoult, and P. R. Mason.** 1991. Isolation of spotted fever group rickettsias from triturated ticks using a modification of the centrifugation-shell vial technique. Trans. R. Soc. Trop. Med. Hyg. **85:**397–398.
31. **Kelly, P. J., P. R. Mason, L. A. Matthewman, and D. Raoult.** 1991. Seroepidemiology of spotted fever group rickettsial infections in humans in Zimbabwe. J. Trop. Med. Hyg. **94:**304–309.
32. **Kelly, P. J., G. Vitale, R. Librizzi, C. Mocciaro, G. Gambino, G. Reina, and P. R. Mason.** 1991. Reactivity of *Rickettsia conorii* monoclonal antibodies with four Zimbabwean spotted fever group isolates. S. Afr. J. Sci. **87:**539–540.
33. **Kelly, P. J., and P. R. Mason.** 1991. Transmission of a spotted fever group rickettsia by *Amblyomma hebraeum* (Acari: *Ixodidae*). J. Med. Entomol. **28:**598–600.
34. **Kelly, P. J., L. A. Matthewman, M. Beati, D. Raoult, P. R. Mason, M. Deary, and J. Makombe.** 1992. African tick-bite fever: a new spotted fever group rickettsiosis under an old name. Lancet **340:**982–983.
35. **Kelly, P. J., L. Beati, L. A. Matthewman, P. R. Mason, G. A. Dasch, and D. Raoult.** 1994. A new pathogenic spotted fever group rickettsia from Africa. J. Trop. Med. Hyg. **97:**129–137.
36. **Kelly, P. J., L. Beati, P. R. Mason, L. A. Matthewman, V. Roux, and D. Raoult.** 1996. *Rickettsia africae* sp. nov., the etiological agent of African tick-bite fever. Int. J. Syst. Bacteriol. **46:**611–614.
37. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
38. **Lange, J. V., and D. H. Walker.** 1984. Production and characterization of monoclonal antibodies to *Rickettsia rickettsii*. Infect. Immun. **46:**289–294.
39. **Li, H., B. Lenz, and D. H. Walker.** 1988. Protective monoclonal antibodies recognize heat-labile epitopes on surface proteins of spotted fever group rickettsiae. Infect. Immun. **56:**2587–2593.
40. **Marrero, M., and D. Raoult.** 1989. Centrifugation-shell vial technique for rapid detection of Mediterranean spotted fever rickettsia in blood culture. Am. J. Trop. Med. Hyg. **40:**197–199.
41. **Maxcey, K. F.** 1929. Endemic typhus fever of the southeastern United States: reaction of the guinea pig. Public Health Rep. **44:**598.
42. **McDade, J. E., C. M. Black, L. F. Roumillat, M. A. Redus, and C. L. Spruill.** 1988. Addition of monoclonal antibodies specific for *Rickettsia akari* to the rickettsial diagnostic panel. J. Clin. Microbiol. **26:**2221–2223.
43. **Nutall, G. H. F.** 1911. On symptoms following tick-bite in man. Parasitology **4:**89–93.
44. **Philip, R. N., E. A. Casper, R. L. Anacker, J. Cory, S. F. Hayes, W. Burgdorfer, and E. Yunker.** 1983. *Rickettsia bellii* sp. nov.: a tick-borne rickettsia, widely distributed in the United States, that is distinct from the spotted fever and typhus biogroups. Int. J. Syst. Bacteriol. **33:**94–106.
45. **Philip, R. N., E. A. Casper, W. Burgdorfer, R. K. Gerloff, L. B. Hughes, and E. J. Bell.** 1978. Serologic typing of rickettsiae of the spotted fever group by microimmunofluorescence. J. Immunol. **121:**1961–1968.
46. **Pijper, A.** 1934. Tick-bite fever. A clinical lecture. S. Afr. Med. J. **8:**551–556.
47. **Pijper, A.** 1936. Etude expérimentale comparée de la fièvre boutonneuse et de la tick-bite fever. Arch. Inst. Pasteur Tunis **25:**388–401.
48. **Pijper, A., and C. G. Crocker.** 1938. Rickettsioses of South Africa. S. Afr. Med. J. **12:**13–30.
49. **Radulovic, S., R. Speed, H. M. Feng, C. Taylor, and D. H. Walker.** 1993. EIA with species-specific monoclonal antibodies: a novel seroepidemiologic tool for determination of the etiologic agent of spotted fever rickettsiosis. J. Infect. Dis. **168:**1292–1295.
50. **Ralph, D., C. Pretzman, N. Daugherty, and K. Poetter.** 1990. Genetic relationships among the members of the family *Rickettsiaceae* as shown by DNA restriction fragment polymorphism analysis. Ann. N. Y. Acad. Sci. **590:**541–553.
51. **Regnery, R. L., C. L. Spruill, and B. D. Plikaytis.** 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J. Bacteriol. **173:**1576–1589.
52. **Roux, V., and D. Raoult.** 1993. Genotypic identification and phylogenetic analysis of the spotted fever group rickettsiae using pulsed-field gel electrophoresis. J. Bacteriol. **175:**4895–4904.
53. **Roux, V., and D. Raoult.** 1995. Phylogenetic analysis of the genus *Rickettsia* by 16S rDNA sequencing. Res. Microbiol. **146:**385–396.
54. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic tree. Mol. Biol. Evol. **4:**406–425.
55. **Sant'Anna, J. F.** 1911. On a disease in man following tick-bites and occurring in Lourenço Marques. Parasitology **4:**87–88.
56. **Uchida, T., T. Uchiyama, and A. H. Koyama.** 1988. Isolation of spotted fever group rickettsiae from humans in Japan. J. Infect. Dis. **158:**664–665.
57. **Uchida, T., X. J. Yu, T. Uchiyama, and D. H. Walker.** 1989. Identification of a unique spotted fever group rickettsia from human in Japan. J. Infect. Dis. **159:**1122–1126.
58. **Uchida, T., T. Uchiyama, K. Kumano, and D. H. Walker.** 1992. *Rickettsia japonica* sp. nov., the etiological agent of spotted fever group rickettsiosis in Japan. Int. J. Syst. Bacteriol. **42:**303–305.
59. **Uchiyama, T., T. Uchida, and D. H. Walker.** 1990. Species-specific monoclonal antibodies to *Rickettsia japonica*, a newly identified spotted fever group rickettsia. J. Clin. Microbiol. **28:**1177–1180.
60. **Urvolgyi, J., and R. Brezina.** 1978. *Rickettsia slovaca*: a new number of spotted fever group rickettsiae, p. 299–305. *In* J. Kazar, R. A. Ormsbee, and I. V. Tarasevich (ed.), Rickettsiae and rickettsial diseases. Proceedings of the Second International Symposium, 1976, Slovak Academy of Sciences. Slovak Academy of Sciences, Bratislava, Slovakia.
61. **Vitale, G., R. D. Stefano, G. Damiani, and S. Mansueto.** 1989. Characterization of Sicilian strains of spotted fever group rickettsiae by using monoclonal antibodies. J. Clin. Microbiol. **27:**1081–1085.
62. **Walker, D. H., Q. H. Liu, X. J. Yu, H. Li, C. Taylor, and H. M. Feng.** 1992. Antigenic diversity of *Rickettsia conorii*. Am. J. Torp. Med. Hyg. **47:**78–86.
63. **Wang, J. G., and D. H. Walker.** 1987. Identification of spotted fever group rickettsiae from human and tick source in People's Republic of China. J. Infect. Dis. **156:**665–669.
64. **Weiss, E., J. C. Coolbaugh, and J. C. Williams.** 1975. Separation of viable *Rickettsia typhi* from yolk-sac and L cell host components by Renografin density gradient centrifugation. Appl. Microbiol. **30:**456–463.