**Rickettsia aeschlimannii** sp. nov., A New Spotted Fever Group Rickettsia Associated with *Hyalomma marginatum* Ticks

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We formally propose the name *Rickettsia aeschlimannii* sp. nov. for a new spotted fever group (SFG) rickettsia, strain MC16, isolated from *Hyalomma marginatum marginatum* ticks collected in Morocco. This organism shows a typical rickettsial morphology when analyzed by electron microscopy. After characterization by serotyping, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western immunoblotting, PCR-restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis, and 16S rDNA sequencing, this organism was found to be different from all of the recognized SFG rickettsiae. Identical PCR-RFLP profiles have, however, been found in *H. marginatum marginatum* from Portugal and *H. marginatum rufipes* from Zimbabwe, which suggests that the distribution of this rickettsia reaches from the Mediterranean to southern Africa.

Although the first descriptions of Mediterranean spotted fever and its ecology were made in northern Africa (13) and one of the reference strains of *Rickettsia conorii* is called the Moroccan strain, there have been few recent reports about the ecology and epidemiology of rickettsioses in this area (21). In early studies, all three African spotted fever group (SFG) rickettsioses, Mediterranean spotted fever, South African tick bite fever, and Kenyan tick typhus (25), were attributed to infection with *R. conorii*. The recognized vectors were dog ticks: *Rhipicephalus sanguineus* in the Mediterranean area (11) and *Rhipicephalus simus* or *Haemaphysalis leachi* in the eastern and southern regions of Africa (22, 27). When other tick species were found to be infected with rickettsia-like organisms (RO), they were considered to be "secondary" vectors of *R. conorii* (21). More recently, however, it has been shown that other SFG rickettsiae occur throughout Africa and that several tick species carry rickettsiae different from *R. conorii* (7, 41).

The presence of RO in *Hyalomma* ticks of Morocco and Sudan was first reported in the 1950s (21). RO have been detected by PCR and direct immunofluorescence in *H. impeltatum*, *H. dromedarii*, and *H. anatolicum* in Egypt; however, they have not been identified (30). During a large-scale field survey in Zimbabwe, hemolymph tests showed that 11% of *Hyalomma marginatum rufipes* ticks were infected with RO (7). Although isolation attempts were unsuccessful, PCR-restriction fragment length polymorphism (RFLP) analysis showed that these ticks contained SFG rickettsiae genotypically similar to a strain (PoTR8) isolated from *H. marginatum marginatum* ticks from Portugal (2). Strain PoTR8 has, however, not been further characterized.

In Morocco, *H. marginatum marginatum* is one of the most widely distributed tick species, possibly representing up to 42% of the tick burden of cattle (3, 32). Although in its juvenile stages it usually bites birds, under particular circumstances it may also infest humans (23). We present here the first description of an SFG rickettsial strain (MC16) isolated from Moroccan *H. marginatum marginatum*. This isolate was compared to the recognized SFG rickettsiae by serotyping, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western immunoblotting, electron microscopy, PCR-RFLP, pulsed-field gel electrophoresis (PFGE), and sequencing of the 16S rDNA and the citrate synthase genes (37, 38). All of these methods indicate that the MC16 isolate is a new SFG rickettsia. We therefore propose the name *Rickettsia aeschlimannii*, in honor of André Aeschlimann, a Swiss zoologist and pathologist who has been very active in tick-related symbionts and pathogens.

**MATERIALS AND METHODS**

Ticks. In March 1992, 40 adult *H. marginatum marginatum* ticks were removed from cattle in the region of Fez, Morocco, and identified according to the usual taxonomic keys (23).

Hemolymph test and isolation attempts. After a 10-min disinfection in iodinated alcohol, the living ticks were rinsed in distilled water and dried on sterile filter paper. One droplet of hemolymph, obtained by cutting a foreleg of the tick (12), was placed on a slide and stained by the method of Giménez (29). A further droplet of hemolymph was mixed with 500 µl of brain heart infusion and inoculated into two shell vials containing monolayers of L929 cells. The vials were centrifuged at 700 × g for 1 h, and the supernatant was discarded and replaced with 1 ml of Earle's minimum essential medium (MEM) containing 4% fetal calf serum and 2 mM L-glutamine (28, 33). After 6 days of incubation in a CO2 incubator at 32°C, the cells were gently scraped from the bottom of the shell vial and stained to detect rickettsiae (20). Cells in infected shell vials were detached with trypsin and transferred to empty 25-cm² flasks, where they were subcultured with MEM.

Rickettsiae and antigen production and purification. The following African and European rickettsial strains were grown in order to compare them with our new isolate: *R. conorii* Moroccan strain ATCC VR-141, *Rickettsia helvetica* ATCC VR-1375, *Rickettsia massiliae* ATCC VR-1376, *R. massiliae* CS (1), *Rickettsia slovaca* 13B, *R. aeschlimanii* ESF-5 (25), and strain Bar29 (10). *Rickettsia slovaca* 13B, *R. aeschlimanii* ESF-5, and *R. helvetica* 13B were kindly provided by G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.). For each reference strain and the new isolate, heavily infected L929 cells from 10 flasks (150 cm²) were harvested, sonicated, and centrifuged at 150 × g for 15 min. The supernatants were layered onto equal volumes of 25% sucrose in PBS (phosphate-buffered saline, pH 7.4) and centrifuged at 7,000 × g for 30 min. The pellets were pooled and purified on a Renografin density gradient (45). Thereafter, the purified organisms were washed in PBS (three times for 10 min each time at 17,500 × g and resuspended in distilled water at a concentration of 1 mg/ml [Total Protein Test: Bio-Rad Laboratories, Richmond, Calif.] for SDS-PAGE and Western blotting. Purification of rickettsiae for PFGE was carried out by following the same procedure, but K26 buffer (16.5 mM KH₂PO₄, 33.5 mM K₂HPO₄, 100 mM KC₃, 15.5 mM NaCl) was used instead of PBS and distilled water (36). The material collected in a further flask was frozen at −80°C until required for mouse immunization, serologic typing, PCR-RFLP analysis, or nucleotide sequence determination.

Transmission electron microscopy. L929 cells were grown to a confluent monolayer in a 5-cm-diameter petri dish with MEM at 37°C in a CO2 incubator. The monolayer was then infected with approximately 10⁷ organisms and resuspended in MEM at 37°C in a CO2 incubator. After a 10-min disinfection in iodinated alcohol, the living ticks were rinsed in distilled water and dried on sterile filter paper. One droplet of hemolymph, obtained by cutting a foreleg of the tick (12), was placed on a slide and stained by the method of Giménez (29). A further droplet of hemolymph was mixed with 500 µl of brain heart infusion and inoculated into two shell vials containing monolayers of L929 cells. The vials were centrifuged at 700 × g for 1 h, and the supernatant was discarded and replaced with 1 ml of Earle's minimum essential medium (MEM) containing 4% fetal calf serum and 2 mM L-glutamine (28, 33). After 6 days of incubation in a CO2 incubator at 32°C, the cells were gently scraped from the bottom of the shell vial and stained to detect rickettsiae (20). Cells in infected shell vials were detached with trypsin and transferred to empty 25-cm² flasks, where they were subcultured with MEM.

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Mouse immunization and microimmunofluorescence serotyping (MIF). According to previously described methods (34), 10^3 to 10^5 organisms each of MC16^T, R. conorii, R. rhipicephali, R. massiliae, Bar29, and R. helvetica were reacted with both homologous and heterologous antigens. Fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulins G and M (Immuno- tion, a (or b), and Ab (or Ba) is the -log, of the endpoint titer of serum A

Anti-R. massiliae

<table>
<thead>
<tr>
<th>Strain</th>
<th>MC16^T</th>
<th>R. massiliae</th>
<th>Strain Bar29</th>
<th>R. helvetica</th>
<th>R. conorii</th>
<th>R. rhipicephali</th>
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PCR-RFLP. DNA was extracted from cells heavily infected with MC16^T, R. massiliae, R. conorii, R. rhipicephali, or Bar29 by using a QiAmp Tissue kit (Qagen, Hilden, Germany). For each strain, cells harvested from a 25-cm^2 flask were lysed by following the producer's instructions and the DNA was eluted into 200 μl of distilled water. A sample containing 10 μl of the extracted DNA, 59.5 μl of distilled water, 10 μl of Taq buffer (Perkin Elmer Cetus, Norwalk, Conn.), 10 μl of deoxynucleotide triphosphates (2% dATP, 2% dCTP, 2% dGTP, and 2% dTTP in distilled water, Boehringer-Mannheim, Munich, Germany) diluted at 1/100 in PBS were used to detect antibodies, and the highest serum dilutions giving positive reactions were recorded as end-point titers. Specificity differences (SPDs) were calculated by following previously described methods (34): SPD = (Aa + Bb) - (Ab + Ba), where Ab (or Br) is the -log, of the endpoint titer between serum A (or B) and the homologous antigen, a (or b), and Ab (or Ba) is the -log, of the endpoint titer of serum A (or B) against its heterologous antigen, b (or a). If the SPD is <3, the two strains are assumed to belong to a single serotype; if the SPD is ≥3, the serotypes are different.

SDS-PAGE and Western blot immunosassay. For each strain, a 5-μl volume of purified organisms was dissolved in 5 μl of Laemmli (29) solution (4% SDS, 10% 2-mercaptoethanol, 0.5% bromophenol blue, 0.125 M Tris hydrochloride [pH 6.8], 25% glycerol) at room temperature and SDS-PAGE was carried out with a 7.5% separating gel and a 3.9% stacking gel. The gel was run in a Mini-Protein II cell (Bio-Rad) at 10 mA in an ice bath, and protein bands were visualized by Coomassie blue staining. A high-range molecular weight standard (Bio-Rad) was used to estimate the molecular weights of the electrophoretic bands. Another, identical gel was transferred (42) to nitrocellulose paper in a Trans-blot apparatus (Bio-Rad) at 50 V for 1 h in an ice bath. Non-specific binding sites were blocked overnight with 5% nonfat dry milk–TBS (10 mM Tris hydrochloride [pH 7.5], 250 mM NaCl, 0.01% merthiolate). After three 10-min washes in TBS, the nitrocellulose paper was overlaid with the polycyclonal serum raised against MC16^T, diluted 1/100 in 3% nonfat dry milk–TBS, and rocked for 2 h. Reactive antibodies were detected with a 1/200 goat anti-mouse globulin-peroxidase conjugate (Immunotech) in 3% nonfat dry milk–TBS. Following three further 10-min washes in TBS, the bound peroxidase was detected by a solution containing 0.015% 4-chloro-1-naphthol, 0.015% hydrogen peroxide, and 16% methanol in TBS. As soon as the bands became visible, the reactions were stopped with repeated washes in distilled water.

FIG. 1. Electron micrograph of MC16^T infecting an L929 cell. R, rickettsia; N, nucleolus; C, trilaminar cell wall; S, translucent zone compatible with the presence of a slime layer. Bar, 0.2 μm.

TABLE 1. MIF antibody titers and SPDs obtained from reciprocal cross-reactions of mouse antisera with SFG rickettsiae

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<td>8,192 (3)</td>
<td>16,384 (0)</td>
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<td>Anti-Bar29</td>
<td>262,144 (6)</td>
<td>1,024 (7)</td>
</tr>
<tr>
<td>Anti-R. helvetica</td>
<td>64 (11)</td>
<td>256 (6)</td>
</tr>
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restriction with BstHII, the migration conditions were 33 h at 4.5 V/cm with ramped pulse times varying from 5 to 120 s. A low number of bands is commonly observed after digestion with BstHII. Therefore, this enzyme is particularly interesting for evaluation of the overall size of rickettsial genomes. To discriminate all of the bands obtained after restriction with Smal and EagI, two different protocols were used for migration of the digests: 19 h at 5.7 V/cm (ramped pulse times from 3 to 10 s) and a further 10 h at 5.4 V/cm (ramped pulse times from 20 to 40 s) or 8 h at 6 V/cm (ramped pulse times from 1 to 3 s) followed by 9 h at 6 V/cm (ramped pulse times from 1 to 5 s). Low-range and lambda ladder PFGE markers (New England BioLabs) were used for estimation of the molecular sizes of the digested products. Computer-aided estimation of the molecular weights was performed as described for PCR-RFLP analysis.

16S rRNA gene sequencing and data analysis. The DNA was extracted from MC16T as described in the PCR-RFLP protocol. The primer pairs used for the amplification and sequencing of the gene encoding the 16S rRNA have been previously described (37). The amplicons were sequenced by using an Autoread automated DNA sequencer (Pharmacia). The sequence of the 16S rRNA gene of strain MC16T was identical to that of the other classical SFG rickettsiae (45, 95, 105, and 135 bp) (Fig. 4). The 190-kDa protein gene fragment of MC16T showed a profile identical to that of R. rhipicephali and strain Bar29. The migration patterns of the cross-reacting bands revealed were different, however, allowing one to distinguish these four rickettsiae (Fig. 3).

PCR-RFLP. After amplification with the citrate synthase primers and digestion with AluI, the profile of MC16T was identical to that of the other classical SFG rickettsiae (45, 95, 105, and 135 bp) (Fig. 4). The 190-kDa protein gene fragment of MC16T showed a profile identical to that of R. rhipicephali when digested with RsaI (95, 106, 118, and 220 bp). Following restriction with PstI, however, the profile was different from classical SFG rickettsiae.

RESULTS AND DISCUSSION

Hemolymph test and isolation attempts. Six of 40 ticks were found to be infected with RLOs. This infection rate of 15% is consistent with the infection rates of H. marginatum nymphs ticks in Zimbabwe (6 to 14%) and H. marginatum marginatum ticks in Portugal. However, in Portugal it has been observed that the infection rate may vary from 11 to 30%, depending on the geographic area (1a). Five isolates were obtained after centrifugation of the hemolymph on shell vials; however, cell lines were found to be contaminated with Mycoplasma spp., and after decontamination (16), only a single isolate, MC16T, could be recovered. The contaminated strains were, however, analyzed by PCR-RFLP and had profiles identical to that of strain MC16T.

Electron microscopy. Strain MC16T is an intracellular organism which occurs free in the cytoplasm of its host cell (Fig. 1). At a higher magnification, MC16T shows typical rickettsial morphology, with a trilaminar cell wall surrounded by a translucent area compatible with the presence of an outer slime layer (Fig. 1). The length of MC16T cells varies from 0.7 to 1.1 pm, and the mean diameter is 0.3 pm.

MIF. Calculated SPDs between strain MC16T and the other considered SFG rickettsiae were all ≥3, indicating that MC16T is a new SFG serotype (Table 1).

SDS-PAGE and Western immunoblotting. Although the MC16T antigenic profile shares some species-specific high-molecular-weight bands with R. massiliae and strain Bar29, its migration patterns are unique and easy to differentiate from those of all other SFG rickettsiae. The approximate molecular masses of the species-specific bands have been evaluated as follows: (116, 120), 140, and 145 kDa for R. rhipicephali; 95, (116), and 150 kDa for MC16T; (116), 135, 140, and 150 kDa for Bar29; 110, (116), 130, (150), and 160 kDa for R. massiliae; 120 and (135) kDa for R. conorii; 120, (138), and 155 kDa for R. afncae; 120, (125), 135, and 140 kDa for R. slovaca; (120), 130, and 155 kDa for R. sibirica; and (90, 120), and 140 kDa for R. helvetica (the more prominent bands are in parentheses) (Fig. 2).

By Western blotting, anti-MC16T antibodies reacted with low-molecular-mass bands (<50 kDa) of each of the rickettsiae. Cross-reactions with high-molecular-weight species-specific antigens were found only with R. massiliae, R. rhipicephali, and strain Bar29. The migration patterns of the cross-reacting bands revealed were different, however, allowing one to distinguish these four rickettsiae (Fig. 3).

FIG. 2. SDS-PAGE of the antigens of R. rhipicephali (lane 1), strain MC16T (lane 2), strain Bar29 (lane 3), R. massiliae (lane 4), R. conorii (lane 5), R. africaca (lane 7), R. slovaca (lane 8), R. sibirica (lane 9), and R. helvetica (lane 10). Standard molecular mass markers, from the bottom, 49.5, 80, 116, and 205 kDa, were in lane 6.

FIG. 3. Western immunoblot assay. Antibodies of anti-MC16T sera reactive with R. rhipicephali (lane 1), strain MC16T (lane 2), strain Bar29 (lane 3), R. massiliae (lane 4), R. conorii (lane 5), R. africaca (lane 6), R. slovaca (lane 7), R. sibirica (lane 8), and R. helvetica (lane 9). The bars on the left correspond to molecular masses (from the bottom, 49.5, 80, 116, and 205 kDa).
that of *R. rhipicephali* (260 and 280 bp) and similar to that of *R. slovaca* and *R. japonica* (125, 165, and 265 bp) (15). This combination of PCR-RFLP migration patterns (Fig. 5 and 6) has already been observed during PCR-RFLP analysis of crude extracts of Zimbabwean *H. marginatum rufipes* (7) and Portuguese *Hyalomma* isolate PoTiR8 (2).

**PFGE.** After restriction of the whole genome with BssHII, the genome size of MC16 is estimated to be 1,336 kb (Fig. 7), suggesting that MC16, like strain Bar29, *R. massiliae*, and *R. helvetica*, is characterized by a genome size slightly greater than that of the other SFG rickettsiae (1,216 to 1,289 kb) (36). PFGE results obtained after restriction with BssHII, SmaI, and EagI show that MC16 is clearly different from other SFG rickettsiae, including the closely related species *R. rhipicephali* and *R. massiliae* and strain Bar29 (Fig. 8).

**16S rRNA gene sequencing and data analysis.** A 1,432-bp sequence, representing 95% of the entire 16S rRNA gene, was generated for MC16 and has been deposited in the GenBank database under accession number U74757. Comparison of 16S rRNA gene sequences derived from SFG species revealed an high level of similarity between strain MC16 and strain Bar29 (99.6%), *R. massiliae* (99.5%), and *R. rhipicephali* (99.6%). As already pointed out (25, 37), the levels of 16S rRNA similarity among SFG rickettsiae are all very high. Between MC16 and the other SFG rickettsiae, similarity varies from 98% (*Rickettsia akari*) to 99.4% (*R. conorii*, *R. japonica*, and *R. slovaca*). Similarly, evolutionary distances between MC16 and the other SFG rickettsiae must also be considered to be very low, varying from $0.0199 \times 10^3$ nucleotides with the more distant species *R. akari* to $0.0042 \times 10^3$ to $0.0049 \times 10^3$ nucleotides with *R. massiliae*, *R. rhipicephali*, and strain Bar29. The tree inferred from the distance matrix (Fig. 9) suggests that strain MC16 clusters with *R. rhipicephali*, *R. massiliae*, strain Bar29, and *R. montana*. Although the topology of this tree has no statistical support (bootstrap values are <80%) and must therefore be considered with the greatest caution, the clustering of MC16 with at least *R. rhipicephali*, *R. massiliae*, and strain Bar29 has been confirmed by a statistically supported phylogenetic analysis based on comparison of citrate synthase gene sequences (GenBank accession numbers for the citrate synthase genes: *R. rhipicephali*, U59721; *R. massiliae*, U59719; strain Bar29, U59720; strain MC16, U59722) (38). Comparison of the citrate synthase genes provides stronger statistical support for the topology of the deeper branches of the tree, including the “MC16 cluster.” Nodes at this branching level are characterized by bootstrap values varying from 95 to 96% (38). Interestingly, by Western blot immunossay, strain MC16, *R. massiliae*, *R. rhipicephali*, and strain Bar29 are the only organisms that have antigenic epitopes in common in the high-molecular-weight protein band.
bacteria whose main hosts and reservoirs are hard-tick species. Although the relationship between rickettsiae and ticks is not necessarily obligately specific, some rickettsiae have never been found in more than one tick species, including strain MC16\(^T\), which has been found to be associated only with *H. marginatum* ticks. Furthermore, there have been no reports of more than one rickettsial strain being harbored by the same tick specimen. Although the possibility of horizontal transmission of rickettsiae through infected vertebrates or cofeeding processes cannot be completely excluded, the fact that rickettsiae are maternally transmitted strongly suggests that rickettsial lineages can be considered to occupy isolated evolutionary niches within specific tick lines. Within this niche, rickettsiae would have scarce opportunity for recombination by genomic exchange with other, related bacteria. As formal criteria for the definition of rickettsial species have yet to be defined, we

### Justification for a new species.

MIF has long been considered the reference method for rickettsial identification and classification (34). This method does not, however, fulfill the polyphasic criteria generally accepted as necessary for the description of new bacterial species (19, 44). Furthermore, compared with other genotypic and phenotypic analysis methods, MIF is a fastidious and sometimes hazardous technique, the use of which can no longer be justified. All other analysis, including SDS-PAGE, Western immunoblotting, PCR-RFLP, PFGE, and 16S rRNA gene sequencing, concurred with the MIF results in indicating that the MC16\(^T\) strain is a new rickettsial pheno- and genotype.

Fragmentary data on DNA-DNA homology among SFG rickettsiae indicate that most of these organisms belong to the same species (43), an observation supported by the high level of similarity observed among SFG rickettsial 16S rRNA gene sequences (37). It has been suggested, however, that bacterial species identification cannot be confidently made on the basis of phylogenetic analysis of 16S rDNA sequences which share a level of similarity of >97% (40). It has also been observed (25) that the species identification criterion of >70% DNA-DNA homology, which was initially proposed for the classification of enterobacteria, may not be suitable to the classification of rickettsiae. Moreover, SFG rickettsiae are strictly intracellular bacteria whose main hosts and reservoirs are hard-tick species.
believe that a polyphasic approach to SFG taxonomy, based on phenotypic, genotypic, and ecological data, is the most suitable (25). On the basis of these considerations, we propose that strain MC16T, isolated from an *H. marginatum marginatum* tick in Morocco, be regarded a separate taxonomic species named *Rickettsia aeschlimannii*.

**Description of Rickettsia aeschlimannii sp. nov.** *Rickettsia aeschlimannii* (ae.schli.man’ni.i, L. gen. n. aeschlimannii, of Aeschlimann, named after Andrk Aeschlimann, a Swiss zoologist, is an obligate intracellular bacterium of the tick *Hyalomma marginatum*, which grows in several cell lines (Vero, 1,929, and Hel). It can be grown in Earle’s MEM supplemented with 4% fetal calf serum and 2 mM L-glutam at 32°C. After Giménez staining, this organism is rod shaped (data not shown). As determined by electron microscopy, this rickettsia has features in common with the other SFG rickettsiae. Mouse antisera against this rickettsia show cross-reactivity against antigens of other SFG rickettsiae. MIF, SDS-PAGE, Western immunoblotting, PFGE, PCR-RFLP, and citrate synthase gene and 16S rDNA sequencing indicated that this rickettsia is clearly different from all other recognized SFG rickettsial species, the most closely related organisms being *R. massiliae*, *R. rhipicephali*, and strain Bar29. No information is available about the possible pathogenicity of this organism for vertebrate hosts. The geographic distribution of this bacterium is likely to correspond to the areas where *H. marginatum* ticks have been analyzed (Morocco, Zimbabwe, and Portugal).

**Type strain.** The type strain MC16T, isolated from *H. marginatum marginatum* in Morocco, is held in the collection of the World Health Organization Collaborative Center for Rickettsial Reference in Marseille, France.

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**REFERENCES**