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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

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In 1990, 17 adult *Rhipicephalus turanicus* ticks were collected in the south of France. Two spotted fever group rickettsiae, Mtu1 and Mtu5, were isolated from the hemolymphs of two of these ticks by the centrifugation shell-vial technique by using HEL cells. These isolates were compared with reference spotted fever group rickettsial serotypes by using three identification methods: microimmunofluorescence serologic typing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and polymerase chain reaction followed by restriction endonuclease fragment length polymorphism analysis. The results obtained by all these techniques showed that Mtu1 and Mtu5 are each previously undescribed rickettsial serotypes. A comparison of the three methods used to identify the isolates led us to the conclusion that, in large-scale epidemiological studies, the simplest way to identify isolates in ticks is to first use the polymerase chain reaction-restriction fragment length polymorphism analysis directly on triturated ticks as a screening method to detect interesting rickettsiae, and then attempt to isolate rickettsiae from ticks for identification by microimmunofluorescence and SDS-PAGE, both of which are time-consuming and expensive to carry out.

In the last several years, new rickettsiae from all over the world have been identified. *Rickettsia japonica* was isolated in Japan (38, 39) and in Zimbabwe, a novel spotted fever group (SFG) rickettsia for Africa was described (19). The distribution of many rickettsiae is also changing. *Rickettsia sibirica* was found in the People's Republic of China (8), *Rickettsia slovaca* was isolated from *Dermacentor marginatus* in France and Switzerland (2), the agent causing a SFG rickettsiosis in the Astrakhan region (Russia) (37) was found to be identical to Israeli tick typhus rickettsia (6), and *Rickettsia rhipicephali* was isolated from *Rhipicephalus sanguineus*, the usual vector of *Rickettsia conorii*, in southern France (7). Improvements in the techniques for the isolation and identification of rickettsiae would greatly facilitate their characterization. For large-scale epidemiological studies, the isolation of rickettsiae from ticks or clinical specimens has been simplified by the introduction of the shell-vial technique (20, 26). The polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis (PCR-RFLP) (34) is arguably a more specific and less laborious method for identifying rickettsiae than microimmunofluorescence (MIF) serologic typing (28) or sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of rickettsial antigens (25). In this study, PCR-RFLP, SDS-PAGE, and MIF serologic typing were used in the identification of two previously undescribed SFG rickettsial isolates and were compared with regard to their simplicity and rational use.

In May 1990, *Rhipicephalus turanicus* ticks were collected in the south of France; five rickettsial strains were isolated from these ticks, but only two could be subcultivated. In this area, *R. turanicus* is one of the widely distributed (15, 30) ticks of the *R. sanguineus* complex (14, 24). Despite its host specificity (cattle, sheep, goats) (9), this tick is well adapted to the suburban biotopes and is able to bite humans and dogs (13, 16). In 1989, rickettsiae were isolated from *R. turanicus* collected in the same region, but the rickettsiae were not identified (26). Thus far, the only rickettsial strain isolated from *R. turanicus* (35), which was cultivated and analyzed by rickettsial toxin neutralization tests in mice (3), was classified as a new serotype. By that method, the isolate seemed to be more closely related to the Thai tick typhus rickettsia than to the other SFG rickettsiae. In this report we describe the isolation and characterization of two previously unknown rickettsiae from *R. turanicus*.

MATERIALS AND METHODS

Ticks. A total of 13 ticks (11 females, 2 males) identified as *R. turanicus* according to standard taxonomic keys (24) were collected in May 1990 in Le Sambuc (Bouches-du Rhône, France) from horses, mostly from the inside of the ears. All the horses belonged to the same owner and were allowed to roam free in the Camargue area. Two other *R. turanicus* females were collected in the same village near sheep pastures by dragging a white bath towel over the vegetation. The last two ticks (females) were collected on vegetation in Villeneuve-lès-Avignon (Vaucluse, France) (Fig. 1).

Hemolymph test and isolation of rickettsiae by the shell-vial method. The ticks were disinfected by immersion in iodated alcohol (10 min) and were then rinsed for 10 min in distilled water under a laminar flow hood and allowed to dry on sterile filter paper. Each tick was initially tested by the hemolymph test (5), in which, by cutting off one foreleg of the tick, a drop of hemolymph was obtained. The drop of

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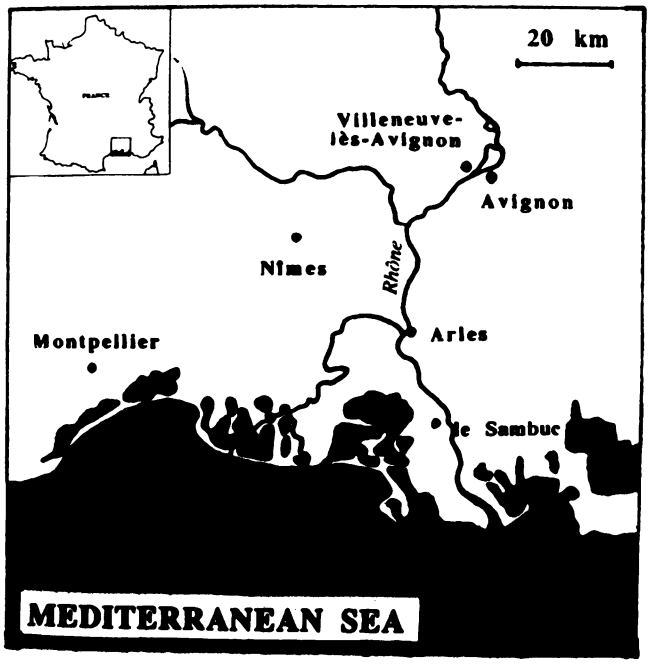

FIG. 1. Geographical localization of the prospected areas in the south of France.

hemolymph was applied to a microscope slide and stained by the method of Gimenez (17). An additional drop was obtained from ticks found to be positive by the hemolymph test, and this was placed in 500 μl of Eagle's minimum essential medium containing 2.2 g of $NaHCO_3$ per liter and 2 mM glutamine (MEM) with 10% fetal calf serum. After mixing, the suspension was divided and added to two shell vials containing an HEL cell monolayer (26). The two shell vials were centrifuged at 700 × *g* for 1 h at 20°C, and the supernatant was discarded. One milliliter of fresh MEM (10% fetal calf serum) was added to each shell vial, which were then incubated at 37°C in 5% CO_2. Six days after inoculation, HEL cells were trypsinized from the shell vials and inoculated into one 25-cm^2 flask containing HEL monolayers. Establishment of the rickettsial strains was obtained through serial subcultures on HEL, Vero, or L929 cells. Infected Vero cell monolayers were observed daily by using an inverted microscope to detect cytopathologic changes.

Electron microscopy. The L929 cell monolayer that was infected by Mtu1 (third day after infection) was fixed in 3.75% glutaraldehyde for 30 min and was then washed in a sucrose-cacodylate buffer for 30 min and fixed again in 1% osmium–1.5% potassium ferricyanide for 1 h. Dehydration was performed by using increasing concentrations of acetone (75 to 100%). The layer was embedded in Araldit, sectioned, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM 1200 EX electron microscope (4).

Rickettsial strains. Isolates made from the shell vials were named M (for Marseille) and tu (for *R. turanicus*) and were numbered according to the number given to each tick when it was collected. The following reference rickettsiae were obtained from the American Type Culture Collection: *R. conorii* Moroccan strain (ATCC VR 141), *Rickettsia rickettsii* Sheila Smith (ATCC VR 149), and *Rickettsia akari* ATCC VR 148). *Rickettsia parkeri* was kindly supplied by D. H. Walker (University of Texas, Galveston); *R. sibirica* 232, *Rickettsia australis*, *R. slovaca*, *R. rhipicephali*, Israeli tick typhus rickettsia, and Thai tick typhus rickettsia were obtained from G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.). *Rickettsia montana*, *R. japonica*, and *Rickettsia helvetica* were not available. All reference strains of rickettsiae were cultivated at 32°C in 150-cm^2 tissue culture flasks on L929 cell monolayers with MEM containing 4% fetal calf serum and 2 mM L-glutamine. Seven days after inoculation, the infected cells were harvested and stored at −80°C.

Mouse immunization. Mouse polyclonal antisera to the SFG reference serotypes and to the new isolates were obtained by the method of Philip et al. (28). Briefly, for each strain, five female Swiss Webster mice were inoculated intravenously via the tail vein on days 0 and 7 with 0.1 ml of infected cells containing about 10^3 to 10^4 PFU. On day 10, the mice were euthanized and exsanguinated, and sera from each group of mice were pooled and stored at −20°C.

MIF. Heavily infected cells of each SFG rickettsia were applied by pen-point at different positions in each well of eight-well microscope slides, air dried, and fixed with acetone. The detection of antibodies in homologous and heterologous antisera was carried out as described by Philip et al. (28) by using fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulins G and M (Immunotec, Marseille, France) diluted at 1/100 in phosphate-buffered saline (PBS; pH 7.2). Twofold dilutions of each serum sample were used to determine the highest titer (endpoint titer) at which rickettsiae could still be detected by MIF. The specificity difference (SPD) was calculated as follows (28): SPD = $(Aa + Bb) - (Ab + Ba)$, where Aa (or Bb) is the antibody titer of serum sample A (or B) against its homologous antigen a (or b), and Ab (or Ba) are the antibody titers of serum sample A (or B) against its heterologous antigen b (or a). In this formula the antibody titers are expressed as $-\log_2$ of the endpoint titer. According to this formula, two strains are different serotypes if the SPD is ≥3, and if the SPD is <3, serotypes are assumed to be identical. Each experiment was done in duplicate, and slides were read independently by two of the investigators.

SDS-PAGE. For each of the 12 rickettsial strains, including the new isolates, the cells in 10 heavily infected 150-cm^2 flasks were harvested with glass beads, sonicated in an ice bath, and centrifuged at 150 × *g* for 15 min. The supernatant was layered onto 15 ml of 25% sucrose (in PBS) and centrifuged at 7,000 × *g* for 30 min. The resultant pellet was purified by Renografin density gradient centrifugation (43). The purified rickettsiae were washed three times in PBS, the final pellet was suspended in distilled water, and the protein concentration was adjusted to 1.0 mg/ml by the Lowry method. For SDS-PAGE, 5 μl of each purified antigen was solubilized in 5 μl of Laemmli (21) solubilizer (4% SDS, 10% 2-mercaptoethanol, 0.5% bromophenol blue, 0.125 M Tris hydrochloride [pH 6.8], 25% glycerol) at room temperature. SDS-PAGE was carried out with a 8% separating gel on a 3.9% stacking gel and run in a Mini-Protean II cell (Bio-Rad, Richmond, Calif.) at 10 mA in an ice bath, and protein bands were visualized by Coomassie blue staining. Low- range and high-range-molecular-weight standards (Bio-Rad) were used to estimate the molecular weights of the electrophoretic bands.

PCR amplification, DNA digestion, and electrophoresis. PCR amplification, DNA digestion, and electrophoresis were carried out as described previously (34). For each of the rickettsiae *R. conorii*, *R. sibirica*, *R. slovaca*, *R. rhipicephali*, *R. parkeri*, Mtu 1, Mtu5, and Mtu11, 1 ml of the

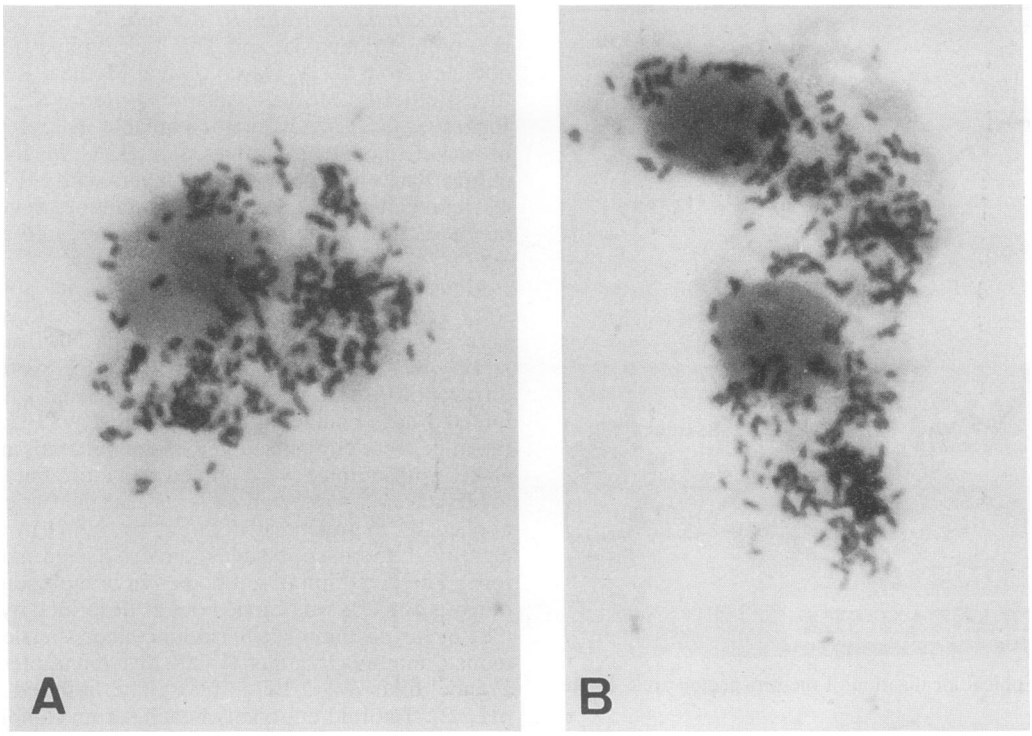

FIG. 2. L929 cells stained by the method of Gimenez (17) and infected with Mtu1 (A) and Mtu5 (B). Magnification, ×1,000.

infected material described above (diluted in distilled water) was centrifuged three times at 17,500 × *g* for 5 min. The final pellet was resuspended in 1 ml of distilled water and boiled for 10 min by the simplified sample treatment procedure recently proposed by Webb et al. (41). A 100-μl reaction mixture containing 10 μl of the boiled suspension, 59.5 μl of distilled H_2O, 10 μl of *Taq* buffer (Boehringer Mannheim, Meylan, France), 10 μl of deoxynucleotide triphosphates (2% dATP, 2% dCTP, 2% dGTP, and 2% dTTP in distilled water; Boehringer Mannheim), 5 μl of each component of the primer pair, and 1 μl of *Taq* polymerase (5,000 U/ml; Boehringer Mannheim) was subjected to 30 repeated cycles of denaturation (95°C for 20 s), annealing (48°C for 30 s), and sequence extension (60°C for 2 min) in a thermal cycler (PREM III Lep Scientific; Flobio, Courbevoie, France). The results of this amplification were visualized on 1.5% agarose (Sigma Chimie, La Verpillière, France) gels after electrophoresis (100 V for 1 h) of 10 μl of the amplified material by staining with ethidium bromide and examination by using UV transillumination. An aliquot of 23.3 μl of the amplification products was then incubated for 2 h at 37°C with 1 μl of the corresponding restriction endonucleases, and the digested products were separated on 8% polyacrylamide gels at 80 V for 4 to 5 h (Bio-Rad), stained with ethidium bromide, and examined by using UV transillumination. DNA molecular weight marker V (Boehringer Mannheim) was run simultaneously with the samples in order to determine the size of the observed DNA fragments (polynomial interpolation of degree 3 of the measured migration data of the standards). PCR-RFLP was performed by using the following oligonucleotide primer pairs (Bioprobe Systems, Montreuil-sous-bois, France) and restriction endonucleases (New England BioLabs, Beverly, Mass.) (34): *Rp*CS.877p and *Rp*CS.1258n (encoding for a 381-bp sequence) with *Alu*I and *Rr*190.70p and *Rr*190.602n (532 bp) with *Rsa*I and *Pst*I.

RESULTS

Isolation of the new strains. Seven of the 17 *R. turanicus* ticks that were collected were positive for rickettsiae by the hemolymph test. All of these positive ticks were females collected from horses in Le Sambuc (Fig. 1). Rickettsiae were isolated from five of these ticks by using shell vials. Three of the isolates, Mtu1, Mtu5, and Mtu11, could be subcultured; but after the infected cells were stored at −80°C for 15 days, only Mtu1 and Mtu5 could be recovered by passage in additional cell cultures. A very small amount (the content of one 25-cm^2 culture flask) of nonviable Mtu11 was available for PCR-RFLP, but there was not a sufficient amount for other studies. Neither Mtu1 nor Mtu5 produced any cytopathologic changes on Vero cell monolayers. Initially, Mtu1 and Mtu5 grew well in HEL, L929, and Vero cells. However, after being frozen at −80°C for 15 days, these isolates could be grown only in HEL cells, which, compared with the other cells, grew slowly. Following two passages in HEL cells, the isolates could again be cultivated in Vero or L929 cells. When infected cells were stained by the method of Gimenez (17), small, intracellular rod or diplobacillary-shaped bacteria could be observed; they were slightly shorter than other SFG rickettsiae (Fig. 2). The new isolates could also be detected in infected cells by indirect MIF by using human serum reactive with *R. conorii* lipopolysaccharide, as previously demonstrated by Western blot (immunoblot) analysis (32).

Electron microscopy. At 3 days after infection, electron microscopy showed that cells had few rickettsiae in their cytoplasms (Fig. 3). The Mtu1 rickettsiae were free in the cytoplasm and were surrounded by a slime layer.

Serological characterization. The relationships of Mtu1 and Mtu5 to the SFG rickettsiae were first analyzed by MIF serologic typing. All 12 serotypes of rickettsiae injected into

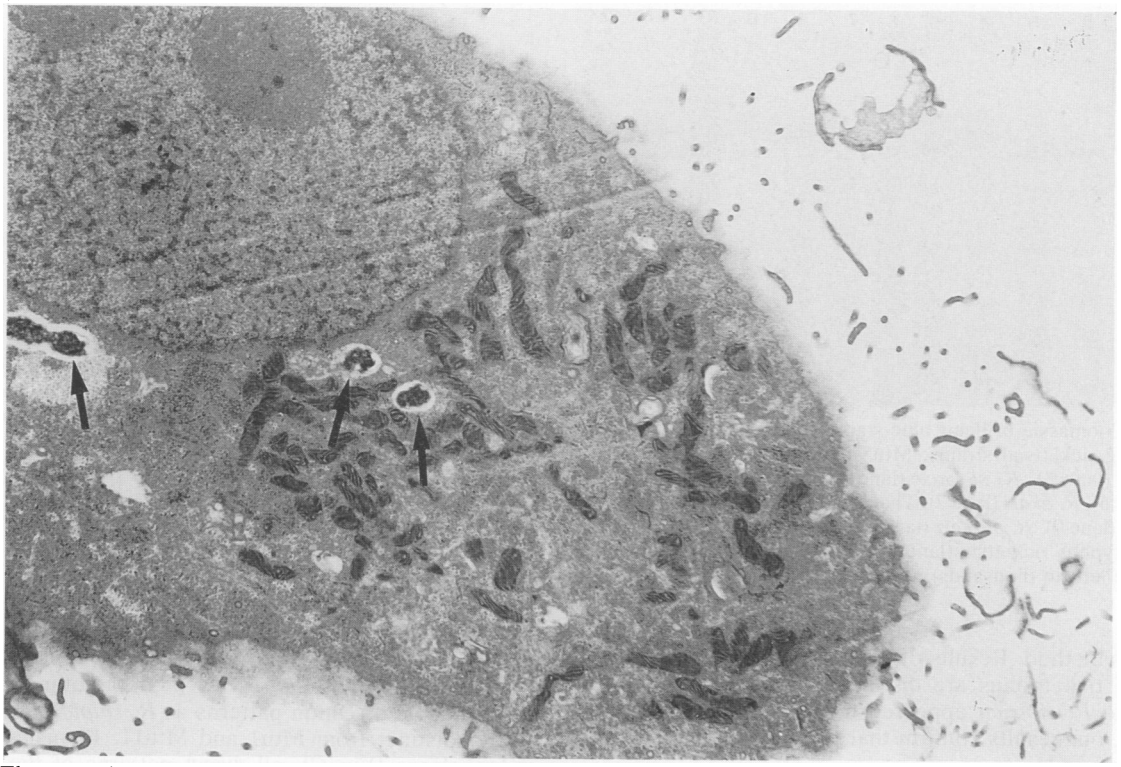

FIG. 3. Electron microscope picture of L929 cells infected with Mtu1 rickettsiae. Rickettsiae are indicated by arrows. Magnification, ×8,000.

mice produced an antibody response detectable by MIF. Endpoint titers and SPDs, which were calculated according to the formula given above, are shown in Table 1. Our results confirm the findings of Philip et al. (28) that comparative MIF can be used to differentiate recognized SFG rickettsiae when SPDs are calculated from homologous and heterologous antibody titers. Homologous titers were usually always at least twofold higher than heterologous titers. However, only a onefold dilution difference between homologous and heterologous titers was observed when the anti-Mtu1 serum reacted against Mtu5 and the anti-*R. slovaca* serum reacted against *R. sibirica*. MIF demonstrated that Mtu1 and Mtu5 are distinct from all the other rickettsial serotypes considered in this study ($13 \geq SPD \geq 6$). The reaction of the anti-Mtu1 and anti-Mtu5 sera to all reference strains was weak, and that of the anti-SFG rickettsiae sera to the new isolates was even weaker (seven SFG antisera did not react with Mtu1 and four did not react with Mtu5). Therefore, by the criteria of Philip et al. (28), Mtu1 and Mtu5 were previously undescribed SFG rickettsial serotypes. In our MIF experiments, we also compared SFG rickettsiae that were not previously compared with reference SFG rickett-

TABLE 1. MIF antibody titers and SPDs from reciprocal cross-reactions of mouse antisera to the new isolates (Mtu1 and Mtu5) and the reference SFG rickettsiae

Antigens	MIF antibody titer (SPD) of mouse antisera to[a]:											
	Mtu1	Mtu5	*R. co.*	*R. rh.*	*R. si.*	*R. sl.*	*R. ak.*	*R. au.*	*R. pa.*	*R. ri.*	IsTT	ThTT
Mtu1	1,024 (0)	64 (5)	<16 (12)	32 (8)	64 (9)	<16 (12)	<16 (13)	<16 (9)	<16 (10)	<16 (11)	<16 (9)	32 (9)
Mtu5	512 (5)	1,024 (0)	16 (10)	32 (6)	64 (9)	32 (9)	32 (10)	<16 (9)	<16 (10)	64 (8)	<16 (9)	<16 (10)
R. conorii	<16 (12)	16 (10)	256 (0)	32 (8)	128 (7)	32 (8)	32 (9)	<16 (8)	16 (6)	128 (4)	32 (6)	64 (6)
R. rhipicephali	64 (8)	256 (6)	16 (8)	512 (0)	128 (9)	16 (11)	16 (9)	<16 (8)	32 (7)	128 (7)	<16 (8)	32 (9)
R. sibirica	32 (9)	32 (9)	16 (7)	<16 (9)	1,024 (0)	256 (3)	16 (12)	16 (8)	32 (5)	64 (5)	16 (5)	16 (7)
R. slovaca	16 (12)	32 (9)	16 (8)	<16 (11)	256 (3)	512 (0)	32 (8)	<16 (8)	16 (7)	32 (6)	16 (7)	32 (6)
R. akari	<16 (13)	16 (10)	<16 (9)	32 (9)	<16 (12)	32 (8)	512 (0)	<16 (9)	<16 (7)	16 (7)	<16 (8)	<16 (9)
R. australis	32 (9)	32 (9)	16 (8)	32 (8)	32 (8)	32 (8)	16 (9)	128 (0)	32 (6)	32 (6)	<16 (8)	<16 (7)
R. parkeri	32 (10)	32 (10)	64 (6)	32 (7)	256 (5)	64 (7)	128 (7)	16 (6)	256 (0)	32 (7)	32 (5)	32 (6)
R. rickettsii	32 (11)	32 (8)	64 (4)	16 (7)	256 (5)	128 (6)	64 (7)	32 (6)	32 (7)	512 (0)	16 (7)	64 (6)
Israeli tick typhus	32 (9)	32 (9)	16 (6)	32 (8)	256 (5)	32 (7)	32 (8)	<16 (8)	32 (5)	32 (7)	128 (0)	32 (5)
Thai tick typhus	16 (9)	32 (10)	16 (6)	<16 (9)	128 (7)	64 (6)	32 (9)	32 (7)	32 (6)	32 (6)	32 (5)	256 (0)

[a] Titers are the reciprocals of the highest dilution of antisera that gave a positive reaction. SPDs are calculated according to the formula given in the text. *R. co.*, *R. conorii*; *R. rh.*, *R. rhipicephali*; *R. si.*, *R. sibirica*; *R. sl.*, *R. slovaca*; *R. ak.*, *R. akari*; *R. pa.*, *R. parkeri*; *R. ri.*, *R. rickettsii*; IsTT, Israeli tick typhus rickettsia; ThTT, Thai tick typhus rickettsia.

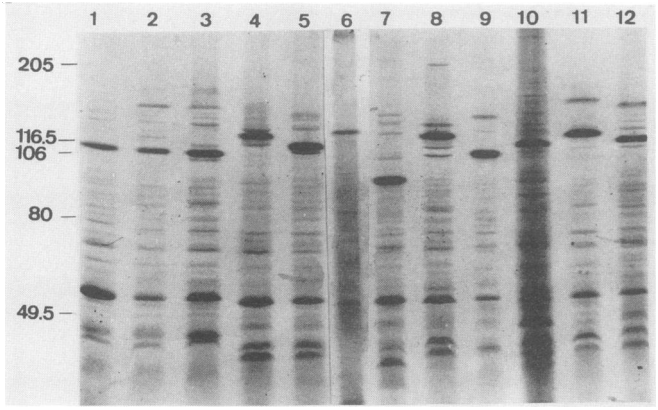

FIG. 4. Coomassie brilliant blue-stained SDS-PAGE profiles of the following rickettsial strains: Mtu5 (lane 1), Mtu1 (lane 2), *R. rhipicephali* (lane 3), *R. slovaca* (lane 4), *R. sibirica* (lane 5), *R. conorii* (lane 6), *R. akari* (lane 7), Thai tick typhus rickettsia (lane 8), *R. australis* (lane 9), *R. parkeri* (lane 10), *R. rickettsii* (lane 11), and Israeli tick typhus rickettsia (lane 12). Molecular weights are indicated on the left (in thousands).

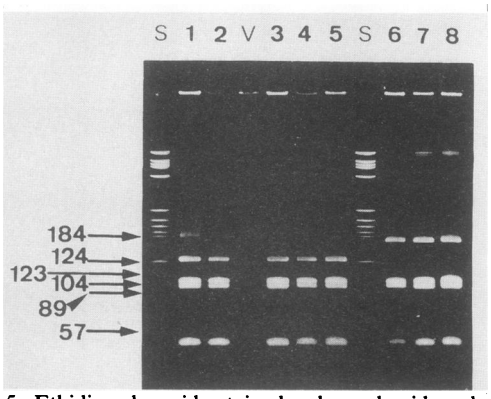

FIG. 5. Ethidium bromide-stained polyacrylamide gels of *Alu*I restriction endonuclease digestion of rickettsial DNA amplified by using the primers *Rp*CS.877p and *Rp*CS.1258n. Lane 1, *R. conorii*; lane 2, *R. parkeri*; lane 3, *R. slovaca*; lane 4, *R. rhipicephali*; lane 5, *R. sibirica*; lane 6, Mtu1; lane 7, Mtu5; lane 8, Mtu11; and S, molecular weight marker V (molecular weights are indicated on the left [in thousands]); lane V, noninfected Vero cells.

siae by this method. Results of these tests showed that Thai and Israeli tick typhus are distinct serotypes. Even if *R. slovaca* and *R. sibirica* appeared to be different serotypes (SPD = 3), our results confirm that they are closely related rickettsiae.

SDS-PAGE. Our SDS-PAGE analyses (Fig. 4) showed that there are multiple similarities between all the rickettsiae in their lower-molecular-mass range (<50 kDa), but the major distinctive proteins were observed in the high-molecular-mass range (>90 kDa). The approximate molecular masses of these distinctive protein bands of the SFG rickettsiae were determined as follows (the most apparent bands are indicated in italic type): 115 and *135* kDa for *R. conorii*; a *135* and a weaker band of 150 kDa band for R. rickettsii; 106, *110*, 135, and 150 kDa for *R. sibirica*; 115, *125*, and 135 kDa for *R. slovaca*; *115* kDa and several weaker bands for *R. parkeri*; *106*, 130, and 145 kDa for *R. australis*; *90*, 100, 135, 140, and 145 kDa for *R. akari*; 106, 110, *130*, 135, and 140 kDa for Thai tick typhus rickettsia; 110, *120*, 135, 140, and 148 kDa for Israeli tick typhus rickettsia; and 100, *110*, 115, *140*, and *150* kDa for *R. rhipicephali*. The new isolates shared two bands (*110* and 140 kDa); however, Mtu1 showed two more antigenic bands (130 and *150* kDa). SDS-PAGE of the new isolates confirmed the MIF findings, that they were new serotypes distinct from one another and from other SFG rickettsiae, even if they shared several bands with other rickettsiae, especially with *R. rhipicephali*.

PCR-RFLP. Results of the PCR-RFLP studies are shown in Fig. 5 to 7. All rickettsiae could be amplified by using the *Rp*CS.877p and *Rp*CS.1258n primers and digestion with *Alu*I restriction endonuclease showed (Fig. 5) that the DNA fragments of the Mtu isolates (Fig. 5, lanes 6 to 8) had migration patterns different from the typical profiles of the SFG rickettsiae. Three bands had sizes identical to those of the SFG rickettsiae (45, 95, and 105 bp), but the Mtu isolates had a high-molecular-mass band of 178 bp instead of 135 bp, as is the case for the SFG rickettsiae. This difference means that the total size of the DNA fragments of the new isolates is 423 bp as opposed to 380 bp for the SFG rickettsiae.

By using the *Rr*190.70p and *Rr*190.602n primer pairs, the amplification products of all rickettsiae showed the same patterns on agarose gels. Restriction of these products with *Pst*I (Fig. 6 and 8) revealed that Mtu1 and Mtu11 had the same profile as that reported for Thai tick typhus rickettsia, *R. rickettsii*, and *R. conorii* 7 (34). On the other hand, Mtu5 had the same digestion patterns as *R. rhipicephali* and was clearly distinct from Mtu1 and Mtu11. By using the *Rsa*I endonuclease (Fig. 7), all digest patterns of the reference rickettsiae corresponded to those reported previously (34), but the new isolates gave one band of about 112 bp and one indistinct undigested patch localized between the 123- and 124-bp bands of the molecular size standard. By using a 16% instead of an 8% polyacrylamide gel and prolonging the migration time by 15 min, it was possible to recognize more than one band at this molecular mass. Increasing amounts of this restriction enzyme did not change the PCR-RFLP pattern for the Mtu isolates (data not shown). When we com-

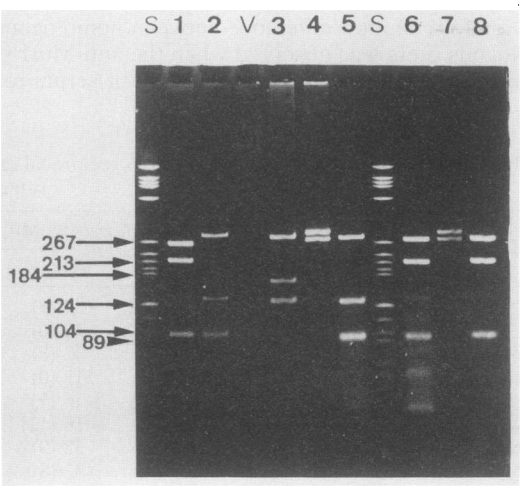

FIG. 6. Ethidium bromide-stained polyacrylamide gels of *Pst*I restriction endonuclease digestion of rickettsial DNA amplified by using primers *Rr*190.70p and *Rr*190.602n. Lane 1, *R. conorii*; lane 2, *R. parkeri*; lane 3, *R. slovaca*; lane 4, *R. rhipicephali*; lane 5, *R. sibirica*; lane 6, Mtu1; lane 7, Mtu5; lane 8, Mtu11; lane S, molecular weight marker V (molecular weights are indicated on the left [in thousands]); lane V, noninfected Vero cells. See Fig. 8 for size estimates of DNA fragments.

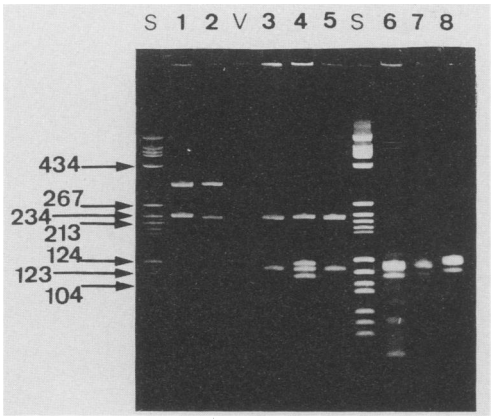

FIG. 7. Ethidium bromide-stained polyacrylamide gels of *Rsa*I restriction endonuclease digestion of rickettsial DNA amplified by using primers *Rr*190.70p and *Rr*190.602n. Lane 1, *R. conorii*; lane 2, *R. parkeri*; lane 3, *R. slovaca*; lane 4, *R. rhipicephali*; lane 5, *R. sibirica*; lane 6, Mtu1; lane 7, Mtu5; lane 8, Mtu11; lane S, molecular weight marker V (molecular weights are indicated on the left [in thousands]); lane V, noninfected Vero cells.

pared our results with previously published data (34) for other SFG rickettsiae, *Rickettsia bellii*, *Rochalimea quintana*, or typhus group rickettsiae, which were not used in our PCR-RFLP analysis, we found the new isolates to be different from the other rickettsiae as well.

DISCUSSION

The SFG rickettsiae are small, gram-negative rod- or diplococcus-shaped obligate intracellular (sometimes intranuclear) bacteria. They live free in the cytoplasm of acarine arthropod or vertebrate cells and are often surrounded by a slime layer (44). Historically, they were first classified according to geographical criteria, and this classification was often supported by distinct clinical features of the diseases that they produced. Serological techniques, like complement fixation (29) or the toxin neutralization test in guinea pigs (3), enabled the differentiation of SFG rickettsiae into serotypes,

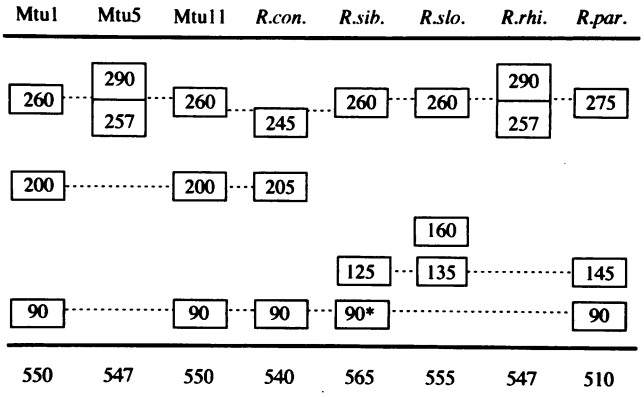

FIG. 8. Schematic electrophoretic migration patterns of PCR-amplified DNA (*Rr*190.70p and *Rr*190.602n) of the considered rickettsiae (Mtu1, Mtu5, and Mtu11, new isolates; *R. con.*, *R. conorii*; *R. sib.*, *R. sibirica*; *R. slo.*, *R. slovaca*; *R. rhi.*, *R. rhipicephali*; *R. par.*, *R. parkeri*) digested with *Pst*I. Doublet bands are indicated with an asterisk. Comigrating fragments are connected by dashed lines. Numbers are molecular weights (in thousands).

serogroups, or strains. Serological typing by MIF with mouse polyclonal antisera (28) is the reference method for identifying SFG rickettsiae. Using this method, Philip et al. (28) described 12 separate serogroups of SFG rickettsiae that were indiscriminately named serotypes or species. Subsequently, SDS-PAGE analyses or immunoblots have provided additional information on the different strains. All of the identification techniques mentioned above have led to an ambiguous classification, in which "strain," "serotype," and "species" are considered taxonomically equivalent terms.

Following the recently proposed criteria (40) based on molecular genetics, two strains belong to the same species if they are characterized by ≥70% (change in thermal denaturation, ≤5°C) relatedness by DNA-DNA hybridization. Another technique for analyzing the phylogenetic relationships between different bacterial strains is the study of the 16S rRNA sequence, but this technique has not always been able to distinguish two species that are well differentiated by DNA hybridization (10). DNA hybridization and 16S rRNA sequencing are not yet routinely used for the identification of SFG rickettsiae. The only strain of SFG rickettsiae analyzed by rRNA sequencing was *R. rickettsii*, which was compared with other members of the family *Rickettsiaceae* and not other SFG rickettsiae (42). So far, DNA hybridization studies show that the SFG rickettsiae (11, 12) are so closely related (at most 4.9% difference in nucleotide sequences) that they can be considered members of a single species. The degree of genetic variability in the SFG rickettsiae is therefore lower than that found normally within a single species of enteric bacteria (11, 31).

Using serological criteria (28) that have so far usually been accepted by rickettsiologists, our new isolates could be considered as new species. We prefer the term "serotype" to the term "species" since, as described above, the use of molecular genetic techniques may lead to the reclassification of the currently recognized species of SFG rickettsiae.

Owing to new detection (1) and isolation (26) techniques, the number of new rickettsial strains in the world is increasing, and the geographical distributions of the classical reference strains are changing. Until 1989, the only rickettsia known in France was *R. conorii*, the agent of Mediterranean spotted fever. Four other rickettsiae have since been described: *R. rhipicephali* (7), *R. slovaca* (2) and the two Mtu isolates. For ecological, epidemiological, and clinical reasons, it is necessary to make an assessment of the rickettsiae present in different areas and their tick vectors. Therefore, large numbers of different ticks will have to be collected and tested for the presence of SFG rickettsiae in the future.

To date, attempts to isolate rickettsiae from hemolymph-positive ticks have been performed without knowing whether such rickettsiae were described previously or whether they were new strains. This blind procedure, however, is both expensive and time-consuming and is not always practical in large-scale epidemiological studies. In addition, although the hemolymph test (5) is a very sensitive technique, it needs to be carried out by experienced technicians and can be used only on living, well-hydrated ticks. The use of PCR-RFLP directly on triturated ticks (1, 41) can detect rickettsiae in frozen ticks, which are easier and safer to store and transport; dehydrated ticks; or ticks preserved in alcohol. In addition, this method not only detects the presence of rickettsiae but also rapidly identifys them. PCR-RFLP is therefore a rapid method for screening new rickettsial strains which need to be isolated for further characterization. Isolation is possible only on living or

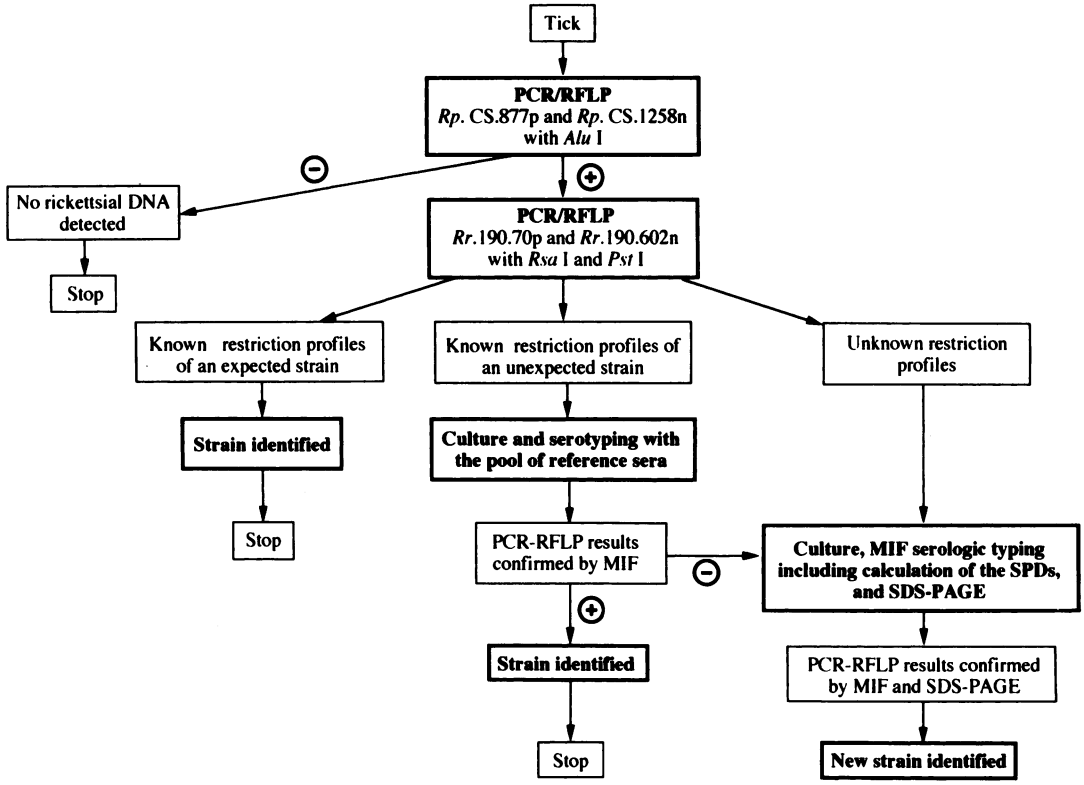

FIG. 9. Proposed algorithm for rickettsial identification during large-scale epidemiological studies.

frozen ticks (−80°C). The identification is possible, however, when the rickettsiae are dead (ticks in alcohol, dehydrated, or frozen at −20°C), so that important epidemiological information can be collected even if the rickettsial strain is not viable for isolation and culture.

The isolation of rickettsiae from ticks has been greatly improved by the introduction of the shell-vial centrifugation assay (20, 26), and the efficacy of this method is confirmed by our results (five strains isolated from seven hemolymph-positive ticks). Following isolation, identification of the rickettsiae can be performed by different methods, all of which have advantages and disadvantages. Of these, MIF requires minimal equipment and only a small amount of rickettsiae (about one 150-cm^2 culture flask for each strain), but the production of antibodies needs laboratory animals and is a laborious, sometimes hazardous, procedure. The culture of sufficient rickettsiae for SDS-PAGE is time- and material-consuming, and the migration patterns of the proteins may be altered by temperature or solubilization conditions (22, 23). On the other hand, the PCR-RFLP technique has the advantage that no culture and purification steps are necessary and only a very small amount of living or killed rickettsiae is required. Unfortunately, the equipment for PCR-RFLP analysis had not arrived in our laboratory at the start of our experiments, and therefore, we were unable to use this method on the ticks directly. It appears, however, that PCR-RFLP is likely the simplest method for determining whether a rickettsia is identical or different from reference strains. The primers and endonucleases recommended (34) for the detection of the SFG rickettsiae are nevertheless not always adapted to the genomic characterization of new strains, as shown by the fact that the *Rsa*I endonuclease could not completely digest the amplified products of the Mtu isolates.

As far as other identification methods are concerned, species-specific monoclonal antibodies are not generally available, and Western blot analysis of strains with polyclonal mouse sera or monoclonal antibodies is characterized by the disadvantages of both MIF and SDS-PAGE. DNA hybridization and rRNA sequencing are not yet in routine use for the identification of SFG rickettsiae.

On the basis of our experiences with the identification of the two new SFG rickettsial serotypes described in this report, we suggest the identification procedure schematized in Fig. 9. This algorithm, which may have to be modified in the future with the development of new identification techniques, may be useful to other researchers who are embarking on large-scale epidemiological projects.

Following isolation and culture, our new rickettsial isolates were identified by three recognized techniques. These isolates were compared with reference SFG rickettsiae and were clearly identified as two new serotypes by each of these techniques. Although three reference rickettsiae—*R. japonica*, *R. montana*, and *R. helvetica*—were absent from our SFG rickettsia reference panel, the published data on these rickettsiae, i.e., SDS-PAGE and immunoblots for *R. japonica* (38, 39) and *R. helvetica* (27) and PCR-RFLP for *R. montana* (34), enabled us to confirm that the Mtu isolates were also different from these three strains. It is not surprising that the three identification methods gave similar results, since all these techniques are based on analysis of the major surface proteins of the rickettsiae (PCR-RFLP also considers the 190-kDa antigen gene). The cross-reactions observed by using polyclonal mouse antisera demonstrate serologi-

cally that the new isolates are related to the SFG rickettsiae. The electron microscope picture (Fig. 3) shows that at 3 days after infection, the number of rickettsiae in the cytoplasm is very low and suggests that the MtuI isolate has an intracellular behavior similar to that of *R. rickettsii* rather than that of the typhus group rickettsiae (36). That result seems to be refuted by the PCR-RFLP profiles obtained by using citrate synthase and *Alu*I endonuclease, which that showed our strains are different from those of the SFG rickettsiae. The restriction profiles obtained by using the 190-kDa antigen primer pair and *Pst*I (specifically used for SFG rickettsial DNA fragments), however, showed migration patterns typical of those of SFG rickettsiae. The 190-kDa antigen primers were unable to amplify rickettsiae of the typhus group—*R. akari*, *R. bellii*, or *R. australis*—which seem to be less related to the SFG rickettsia than the Mtu isolates are (31, 34).

Epidemiologically, it is important that *R. turanicus* ticks can become infected with SFG rickettsiae, because this species of tick has had to adapt to the urbanization of rural areas (13, 16) by feeding more frequently on dogs and humans. Since no strain other than *R. conorii* in this area has ever been isolated from humans, nothing is known about the possible pathogenicity of the new isolates for humans. Also, since *R. turanicus* harbors the new SFG rickettsiae and feeds on humans and since human antisera to SFG rickettsiae cross-react with each serotype of the group (18), the presence of such antibodies against the new isolates could confuse the results of serosurveys, resulting in the misinterpretation of the serological prevalence of Mediterranean spotted fever (33).

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