JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1996, p. 2688–2694 Vol. 34, No. 11
0095-1137/96/$04.00+0

Phenotypic and Genotypic Characterization of Spotted Fever Group Rickettsiae Isolated from Catalan *Rhipicephalus sanguineus* Ticks

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Received 1 April 1996/Returned for modification 28 June 1996/Accepted 20 August 1996

Eighty-nine *Rhipicephalus sanguineus* ticks and 21 *Rhipicephalus bursa* ticks collected in Catalonia were tested by the hemolymph test to establish their infection rate with spotted fever group rickettsiae. By Giménez staining, 11.2% of the *R. sanguineus* isolates and 0% of the *R. bursa* isolates were found to contain rickettsia-like organisms. Six spotted fever group rickettsial strains (Bar29, Bar31, Gir4, Tar1, Tar2, and Tar3) were isolated from these ticks and were characterized by phenotypic and genotypic analyses. PCR followed by restriction fragment length polymorphism analysis showed that the six strains were identical and were characterized by the same restriction profiles as a strain, Mtu5, previously isolated from *Rhipicephalus turanicus* ticks in the South of France. Microimmunofluorescence serotyping, sodium dodecyl sulfate—polyacrylamide gel electrophoresis of the purified organisms, and Western blot (immunoblot) assay with mouse polyclonal sera confirmed this observation. Pulsed field gel electrophoresis of the whole genome of three of the strains showed that, although closely related, the profile of Tar1 was slightly different from that of the Bar strains. Phylogenetic analysis showed that this new rickettsial sero- and genotype, which will be named the "Catalan strain," is closely related to *Rickettsia massiliae*. This strain shows an unexpected resistance to rifampin. The epidemiological implications of these findings are considered.

Rickettsia conorii is the causative agent of Mediterranean spotted fever (MSF) in the Mediterranean area, Middle East, Africa, and Asia. In the Mediterranean area, this organism is transmitted by the brown dog tick, *Rhipicephalus sanguineus*, which plays the epidemiological role of vector and main reservoir.

In Spain, several cases of the disease have been described from all over the country, and serosurveys have been conducted in different areas (16, 19, 20, 22, 25, 31, 42, 44, 45). These studies indicated that epidemiological data recorded in the Catalan region differ from those recorded in other Spanish or Mediterranean regions. For instance, these surveys showed that the seroprevalence of antibodies reactive with spotted fever group (SFG) rickettsiae in human and canine populations was significantly lower in Catalonia than in the center of Spain (16, 26). Catalan patients presented mild forms of MSF compared with those reported from other regions (43, 46). Furthermore, children infected with SFG rickettsiae in Catalonia showed an unusual unresponsiveness to treatments with rifampin (8). The reasons for these epidemiological peculiarities have not been established yet.

The presence of different SFG rickettsial strains, characterized by different levels of pathogenicity and immunogenicity, could explain this situation. In 1989, two rickettsial strains were isolated on shell vials from the blood of patients with MSF contracted in the region of Barcelona (17). They did not produce any cytopathogenic effects on Hel cells, which is in contrast with the growth patterns of *R. conorii* in this cell line (17). However, by pulsed field gel electrophoresis (PFGE), one of them, the 16B strain, has been found to be identical to *R. conorii* (39). The only tick isolate obtained in Spain by Vero cell inoculation with eggs of *R. sanguineus* from dogs near Madrid has been identified, by PCR-restriction fragment length polymorphism (PCR-RFLP) only, as *Rickettsia rhipicephali* (23). SFG rickettsiae genotypically different from *R. conorii* have been detected in or isolated from the hemolymph of ticks belonging to the *R. sanguineus* complex in other Mediterranean countries (1–3, 7, 14). For a better understanding of the epidemiological situation found in Catalonia, it therefore seems necessary to isolate more rickettsial strains from ticks and humans and to compare them with those found in the rest of the Mediterranean area.

In 1992 and 1993, 89 *R. sanguineus* ticks were collected from dogs and 21 *Rhipicephalus bursa* ticks were collected from wild goats (*Capra hispanica*) in different Catalan areas. Hemolymph tests for the detection of rickettsiae and isolation attempts were performed with these arthropods. We present here the phenotypic and genotypic characterization of the strains of SFG rickettsiae isolated from this sample of ticks.

MATERIALS AND METHODS

Tick sampling. Adult *R. sanguineus* ticks were collected from dogs in Vallés Occidental, Pedralta, Sant Jordi Desvalls, and Roquetes in 1992 and 1993, and adult *R. bursa* ticks were collected from wild goats in La Sènia in 1993 (Fig. 1). The ticks were identified according to the usual taxonomic keys (18, 32).

Hemolymph test and isolation of rickettsiae. 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 (10), was placed on a slide and stained by the method of Giménez (21). A further droplet of hemolymph was mixed with 500 μl of brain heart infusion broth 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 (29, 34). After 6 days of incubation in a CO_2 incubator at 32°C, the cells were gently scraped from the bottom of the shell vial and stained to detect rickettsiae (21). Cells in infected shell vials were detached with trypsin and transferred to 25-cm^2 empty flasks, where they were subcultured with MEM (4% fetal calf serum).

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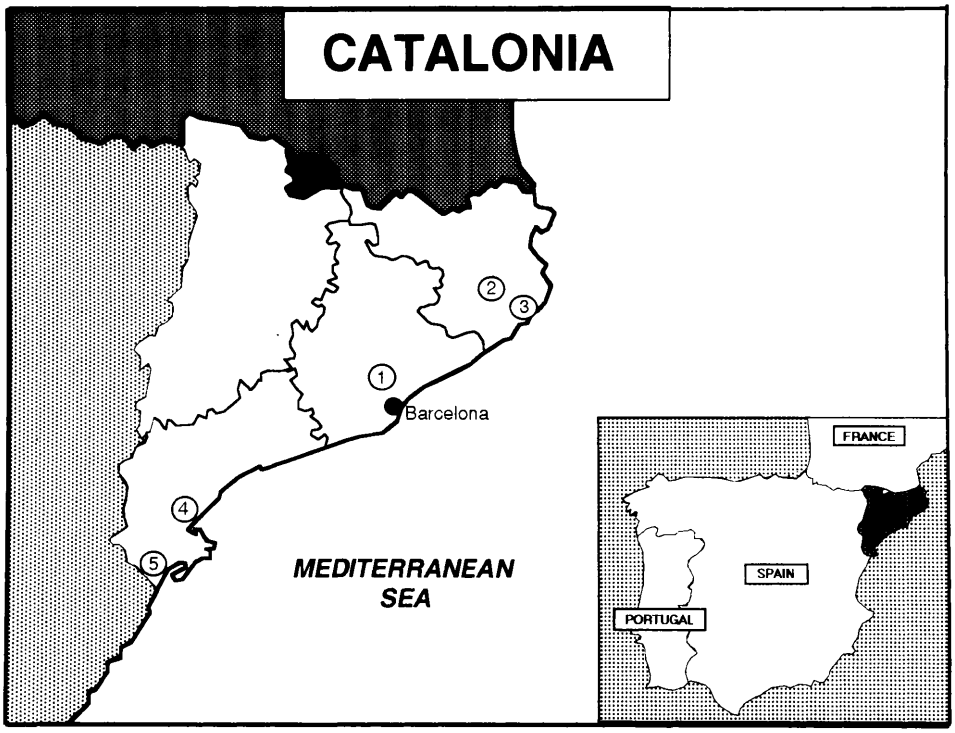

FIG. 1. Map of Catalonia. The sites where the ticks were collected are marked by numbers: 1, Collserola, Vallés Occidental; 2, Pedralta, Girona; 3, Sant Jordi Desvalls; 4, Roquetes; and 5, La Sènia.

Rickettsiae, antigen production, and purification. The following Mediterranean and European rickettsial strains were grown in order to compare them with our new isolates: *R. conorii* (Moroccan strain, ATCC VR-141), *Rickettsia helvetica* (ATCC VR-1375), *Rickettsia massiliae* (ATCC VR-1376), *Rickettsia sibirica* (ATCC 232), the "GS" Greek strain (1), and (kindly provided by G. A. Dasch) *Rickettsia slovaca*, the Israeli spotted fever rickettsia, and *R. rhipicephali*. The Mtu5 strain, which had been isolated from *Rhipicephalus turanicus* ticks, has since been lost (3). However, sera and purified antigen were still available for comparison. For each reference strain and the new isolates, heavily infected cells from 10 flasks (150 cm^2) were harvested, sonicated, and centrifuged at 150 × *g* for 15 min. The supernatants were layered onto equal volumes of 25% sucrose in phosphate-buffered saline (PBS [pH 7.4]) and centrifuged at 7,000 × *g* for 30 min. The pellets were pooled and purified on a Renografin density gradient (49). Thereafter, the purified organisms were washed in PBS (three times, 10 min at 17,500 × *g*) and resuspended in distilled water at a concentration of 1 mg/ml (total protein test; Bio-Rad, Richmond, Calif.) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). The purification of rickettsiae for PFGE was carried out by the same procedure, but K36 buffer (16.5 mM KH_2PO_4, 33.5 mM K_2HPO_4, 100 mM KCl, 15.5 mM NaCl) was used instead of PBS and distilled water (39). The material collected in a further heavily infected flask was frozen at −20°C until used for mouse immunization, serologic typing, and PCR-RFLP.

PCR-RFLP. The new isolates were first analyzed by PCR-RFLP. DNA from cells heavily infected with *R. massiliae*, *R. conorii*, *R. rhipicephali*, and the Catalan isolates was extracted by the QAmp tissue kit method (Qiagen, Hilden, Germany) and used for PCR-RFLP. 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% dTTP, and 2% dGTP in distilled water [Boehringer Mannheim, Meylan, France]), 5 μl of the citrate synthase primer pair *Rp*. CS.877 p-1258n (Appligene, Illkirch, France) or 5 μl of the 190-kDa protein gene primer pair *Rp*. 190.70 p-602n (Appligene) and 0.2 μl of *Taq* polymerase (Perkin-Elmer Cetus, Branchburg, N.J.) was subjected to 35 cycles of denaturation (95°C for 20 s), annealing (48°C for 30 s), and sequence extension (72°C for 2 min) in a thermal cycler (PREM III Lep Scientific; Flobio, Courbevoie, France). A further 5-min extension at 68°C completed the enzymatic polymerization. The results of amplification were visualized on a 1% agarose gel after electrophoretic migration (100 V for 30 min) of 10 μl of the amplified material. The agarose gels were stained with ethidium bromide and examined by UV transillumination. According to previously described protocols, 23.3 μl of the products amplified with the citrate synthase primers (or the 190-kDa protein gene primers) was then digested with 1 μl of restriction endonuclease *Alu*I (or *Rsa*I and *Pst*I) (Boehringer Mannheim). 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 UV transillumination. DNA molecular weight marker V (Boehringer-Mannheim) and computer-aided densitometry were used to estimate the size of the observed DNA fragments (QGEL-DTM program [Quantigel Corporation, Madison, Wis.] connected with "The Imager" system of Appligene). With this system, profiles could be compared with those described in other reports (3–7, 12, 14, 28, 37) and with the computerized data collected in the data bank of the Unité des Rickettsies in Marseille, France.

Mouse immunization and MIF. Considering the results obtained by PCR-RFLP, only the following rickettsial species were used for comparison by serotyping: *R. conorii*, *R. rhipicephali*, *R. massiliae*, and the Mtu5 strain. According to previously described methods (35), 10^3 to 10^4 organisms of each of the rickettsiae mentioned above and the newly isolated strains were inoculated intravenously into five Swiss mice on days 0 and 7. The mice were anesthetized and exsanguinated by cardiac puncture on day 10, and the sera of each group of five mice were pooled and stored at −20°C. For microimmunofluorescence serotyping (MIF), twofold dilutions in PBS of the mouse polyclonal sera reacted to *R. massiliae*, *R. rhipicephali*, *R. conorii*, Mtu5, or the Catalan isolates. Fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin G and immunoglobulin M (Immunotec, Marseille, France) 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 were calculated by previously described methods (35).

SDS-PAGE and Western blot assay. For each of the following isolates of *R. conorii*, *R. rhipicephali*, *R. slovaca*, *R. sibirica*, the Israeli SFG rickettsia, *R. massiliae*, and one of the Catalan strains (Bar29), 5 μl of purified organisms was dissolved in 5 μl of Laemmli (30) 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 (48) to nitrocellulose paper in a Trans-blot apparatus (Bio-Rad) at 50 V for 1 h in an ice bath. Nonspecific 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 antisera to Bar 29, diluted 1/100 in 3% nonfat dry milk–TBS, and rocked for 2 h. Reactive antibodies were detected with 1/200 goat anti-mouse globulins–peroxidase conjugate (Immunotec) in 3% nonfat dry milk–TBS. After three further 10-min washes in TBS, the bound peroxidase was

TABLE 1. Results of tick sampling, hemolymph tests, and isolation attempts

Sampling location	No. of ticks		No. (%) of ticks hemolymph test positive	No. of rickettsia isolates obtained
	R. sanguineus	*R. bursa*		
Sant Jordi Desvalls	10		0 (0)	0
Pedralta, Girona	19		1 (5.3)	1
Collserola, Barcelona	50		6 (12)	2
Roquetes, Tarragona	10		3 (30)	3
La Sènia		21	0 (0)	0

detected with 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. All Catalan strains, *R. massiliae*, and the Mtu5 strain were used for a further electrophoresis and Western immunoblotting according to the same protocol.

PFGE. Each of the bacterial suspensions of Bar29, Bar31, and Tar1; *R. massiliae*; and the Greek "GS" strain was added to 1 volume of Incert agarose (FMC Bioproducts, Rockland, Maine) to form agarose blocks and lysed as previously reported (39). Thereafter, the plugs were digested with the following endonucleases: *Sma*I (Boehringer Mannheim, Illkirch, France), *Eag*I (New England BioLabs, Beverly, Mass.), and *Bss*HII (New England BioLabs). The migrations were carried out in a contour-clamped homogeneous electric field system (CHEF DRII; Bio-Rad Laboratories) at 14°C in 0.5× Tris-borate-EDTA (pH 8). After restriction with *Bss*HII, the migration conditions were as follows: 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 *Bss*HII. Therefore, this enzyme is particularly interesting for the evaluation of the global size of rickettsial genomes. In order to discriminate all of the bands obtained after restriction with *Sma*I and *Eag*I, two different protocols were used for the migration of the digests: 19 h at 5.7 V/cm (ramped pulse time from 3 to 10 s) and 10 further h at 5.4 V/cm (from 20 to 40 s) or 8 h at 6 V/cm (from 1 to 3 s) followed by 9 h at 6 V/cm (from 1 to 5 s). Low-range and lambda ladder pulsed field gel markers (New England BioLabs) were used for the estimation of the molecular size of the digested products. The computer-aided estimation of the molecular weights was performed as described for the PCR-RFLP.

In vitro activity of rifampin. The Catalan isolates did not produce cytopathogenic changes in cell monolayers. Therefore, classical microplaque colorimetric assays (36) could not be performed and had to be replaced by the following procedure. Monolayers of Vero cells were grown into 96-well microplaques in MEM and infected with the Bar29 strain (100 organisms per well). After a 1-h incubation at room temperature, the culture medium was replaced by serial dilutions of rifampin in MEM (from 0.0625 to 2 μg/ml). From the 3rd to the 12th day after infection, cells were gently scraped at the bottom of each well and stained by the Giménez method in order to detect rickettsiae and to compare the infection rate with that of a control plaque, where rickettsiae were grown in MEM without antibiotics.

RESULTS

Tick sampling, hemolymph test, and isolations. Eighty-nine (41 females and 48 males) *R. sanguineus* ticks and 21 (12 females and 9 males) *R. bursa* ticks could be recovered alive from a sample of 130 *R. sanguineus* ticks and 30 *R. bursa* ticks after storage at 28°C under saturated conditions of humidity for 1 month. They were collected in the different Catalan areas shown in Fig. 1. Each tick was named according to the geographical origin and numbered. The ticks from Collserola were called Bar (for Barcelona), those from Pedralta were called Gir (for Girona), and those from Roquetes were called Tar (for Tarragona).

By the hemolymph test, 10 *R. sanguineus* ticks (4 females and 6 males) and none of the *R. bursa* ticks were found positive for infections with rickettsia-like organisms. A total of six strains could be isolated and subcultured: Bar29, Bar31, Gir4, Tar1, Tar2, and Tar3. All of the new strains were found to be contaminated with mycoplasmas and were purified by inoculation into BALB/c mice, and reisolation of rickettsiae from the brains or the spleens of the mice (13) was accomplished after 3 to 5 days. After the procedure, Gir4 was lost. All of these strains grew well in L929, Hel, or Vero cells and did not have any cytopathogenic effect on the cell monolayers. Subcultures could be carried out by subsequent trypsinizations of the monolayers. The results of the hemolymph tests and isolation attempts are reported in Table 1.

PCR-RFLP. With the primers and endonucleases described above, the profiles of the six isolates were always identical (data not shown). The restriction profiles of the Catalan strains obtained after amplification with the citrate synthase primer pair were identical to those previously described for *R. massiliae* and the Mtu5 strain (45, 95, 105, and 178 bp) and different from those of other SFG rickettsiae (45, 95, 105, and 135 bp) (Fig. 2). The amplification with the 190-kDa protein gene primer pair allowed us to determine that the new isolates have the same profiles as those of *R. massiliae* after digestion with *Rsa*I (99, double 111, and double 116 bp) (Fig. 3) and *R. rhipicephali* when digested with *Pst*I (270 and 290 bp) (Fig. 4), which is consistent with data recorded for the Mtu5 strain (3).

MIF. All sera to the new isolates reacted with their homologous antigen, with the other Catalan isolates and the Mtu5 strain at almost the same titers, with the recorded differences never being greater than a twofold dilution (Table 2), and with the specificity differences never being higher than 2. The titers obtained against *R. massiliae*, *R. rhipicephali*, and *R. conorii* were clearly lower, and specificity differences varied from 4 to 12. MIF confirmed that the Catalan serotypes were identical to the Mtu5 strain. Specificity differences between the Mtu5

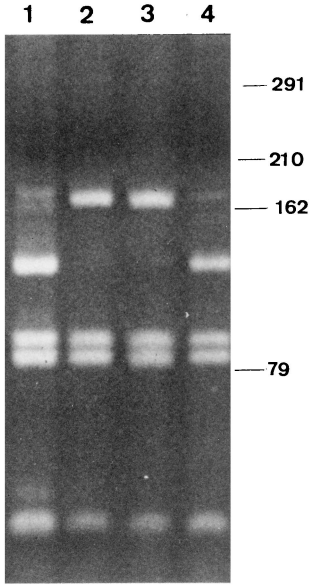

FIG. 2. Ethidium bromide-stained profiles of DNA fragments of *R. conorii* (lane 1), *R. massiliae* (lane 2), Bar29 (lane 3), and *R. rhipicephali* (lane 4) amplified with the citrate synthase primers and digested with *Alu*I. Standard molecular sizes marked on the right in base pairs.

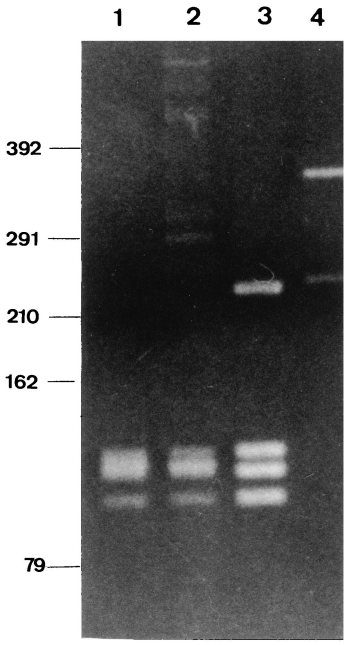

FIG. 3. Ethidium bromide-stained profiles of DNA fragments of *R. massiliae* (lane 1), Bar29 (lane 2), *R. rhipicephali* (lane 3), and *R. conorii* (lane 4) amplified with the 190-kDa protein primers and digested with *Rsa*I. Standard molecular sizes are marked on the left in base pairs.

strain and other SFG rickettsiae have been previously reported (3, 6).

SDS-PAGE and Western blot immunoassay. The electrophoretic migration patterns of the new strains were very similar to each other and to those of the Mtu5 strain (data not shown)

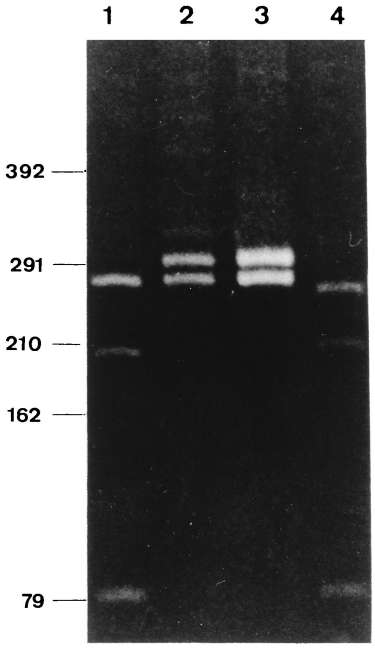

FIG. 4. Ethidium bromide-stained profiles of DNA fragments of *R. massiliae* (lane 1), Bar29 (lane 2), *R. rhipicephali* (lane 3), and *R. conorii* (lane 4) amplified with the 190-kDa protein primers and digested with *Pst*I. Standard molecular sizes are marked on the left in base pairs.

(3). The high-molecular-mass bands of Bar29 were localized at 116, 135, 140, and 150 kDa and differed from those of all other European and Mediterranean SFG rickettsiae. The major high-molecular mass bands of *R. conorii* had approximate sizes of 120 and 135 kDa. The bands were 110, 116, 130, 150, and 160 kDa for *R. massiliae*; 116, 120, 140, and 145 kDa for *R. rhipicephali*; 120, 138, and 155 kDa for the Israeli spotted fever rickettsia; 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* (Fig. 5). By Western immunoblotting, antibodies of the anti-Bar29 sera cross-reacted with low-molecular-mass antigenic bands (<50 kDa) of all of the other SFG rickettsiae; however, they did cross-react only with the high-molecular-mass specific proteins of *R. rhipicephali* and *R. massiliae* (Fig. 6). The second Western blot showed that the antibodies of the anti-Bar29 sera reacted similarly with Bar29, Bar31, Tar1, Tar2, Tar3, and Mtu5, whereas their reactions with *R. massiliae* were located on bands with slightly different molecular weights. Although the concentration of the Mtu5 antigen was lower than that of the other strains, it was evident that its profile was identical to those of the Catalan strains (Fig. 7).

PFGE. Three strains were tested by PFGE: Bar29, Bar31, and Tar1. After digestion of their genome with different restriction enzymes, the profiles of these isolates were clearly distinct from those of all previously described SFG rickettsiae (39). With *Eag*I, the three Catalan isolates showed identical migration patterns; with *Sma*I, the profile of Tar1, however, was characterized by an additional band, which would suggest that there are slight genomic differences among these strains (Fig. 8). With *Bss*HII, the approximate genome size of these isolates was 1,365 kb, which is slightly higher than that of the other SFG rickettsiae but consistent with that of *R. massiliae* (38, 39).

In vitro activity of rifampin. Living Bar29 rickettsiae were found in all of the wells from the 3rd to the 12th day after infection, despite the presence of rifampin at increasing concentrations. The cells in all wells were heavily infected, and their infection rate did not differ from that observed in the control plaque. Therefore, rifampin did not seem to be effective against Bar29 in vitro.

DISCUSSION

Reports dealing with the resurgence of MSF in Spain in the early 1980s (31, 33, 45) have been behind the origin of several clinical studies of the disease and serosurveys in humans and dogs (16, 20, 22, 25, 31, 42–46). So far, the two strains isolated in Spain have been identified as *R. conorii* in Catalonia and *R. rhipicephali* in the region of Madrid (17, 23, 39). For a better knowledge of the epidemiology of SFG rickettsioses in Spain, more rickettsial isolates are needed in order to identify them and compare them with reference strains. Therefore, we started to analyze Catalan *R. sanguineus* ticks, the tick species which is more likely to bite humans in that region. The five isolates obtained from ticks collected in different Catalan areas are consistently identical according to MIF, SDS-PAGE, Western blots, and PCR-RFLP. Only PFGE could detect a minor difference in the restriction profiles of one of these isolates. Compared with PCR-RFLP results recorded in our database, these isolates seem to be identical to the Mtu5 strain (3). SDS-PAGE, Western blotting, and MIF confirmed this result. The Mtu5 strain was isolated from *R. turanicus* in the South of France (3) and had been lost thereafter. Previously published 16S rRNA gene sequencing data (40) as well as other genotypic and phenotypic analyses confirm that this rickettsia is closely related to *R. massiliae* and *R. rhipicephali*, which are

TABLE 2. MIF serotyping of the Catalan isolates used in this study

Antigen	Reciprocal value of end point titer of:									
	R. conorii	*R. rhipicephali*	*R. massiliae*	Mtu5	Bar29	Bar31	Gir4	Tar1	Tar2	Tar3
R. conorii	1,024	256	32	64	1,024	2,048	256	128	128	64
R. rhipicephali	64	4,096	512	128	4,096	4,096	512	1,024	512	512
R. massiliae	64	512	2,048	64	2,048	4,096	512	128	128	256
Mtu5	32	512	512	512	8,192	8,192	2,048	2,048	1,024	8,192
Bar29	32	256	256	256	16,384	16,384	2,048	2,048	1,024	4,096
Bar31	32	128	256	512	16,384	16,384	2,048	2,048	2,048	8,192
Gir4	32	128	512	256	8,192	8,192	2,048	2,048	2,048	8,192
Tar1	32	128	256	256	8,192	8,192	2,048	2,048	1,024	8,192
Tar2	32	128	256	512	16,384	8,192	1,024	2,048	1,024	4,096
Tar3	32	128	256	512	8,192	16,384	2,048	2,048	2,048	8,192

both related to ticks of the *R. sanguineus* complex. Similarity rates between SFG rickettsiae are very high, usually >97%. For Bar29, they vary from 99.9% with *R. massiliae*, the more closely related strain, to 98.3% with *R. akari*, the more distant strain. It has been shown, however, that (40) phylogenetic reconstructions of the SFG group, on the basis of 16S rRNA gene analysis, are not statistically supported. Reconstructions based on the divergence of the citrate synthase gene have thereafter proven to be more reliable. Bootstrap values >95% confirm that Bar29 rickettsiae belong to a cluster containing *R. massiliae* and *R. rhipicephali*. With the citrate synthase gene, similarity rates between Bar29 vary from 99.8% with *R. massiliae* to 94.2% with *R. akari* (41).

From an epidemiological point of view, it is important to emphasize that, so far, we have not isolated *R. conorii* from Catalan *R. sanguineus*, although MSF is considered to be endemic in Catalonia.

According to the literature, in Spain serological prevalences of antibodies reacting with *R. conorii* antigens in humans and dogs seem to differ from one region to another. By the indirect immunofluorescence test at a cutoff titer of ≥1:40, the prevalence of antibodies reacting with *R. conorii* antigens in human sera varied from 11.6% in Catalonia (16) up to 73.5% in the region of Salamanca (26). Indirect immunofluorescence detected antibodies to *R. conorii* in 37% of the sera collected from dogs in the area of Barcelona (Catalonia) (16) and in 58.6% of those collected in the central provinces of Spain (22) (titer of ≥1:40). A variability in the prevalence of antibodies reactive with *R. conorii* was, however, observed in a dog population of Catalonia, the prevalence of antibodies reactive with *R. conorii* was, however, observed in a dog population of Catalonia, the prevalence being significantly higher in summer (37%) than in winter (<1%) (15, 16). Similarly, it seems that there are more severe and fatal forms of MSF in the region of Salamanca (42) than in Catalonia (46).

Three main observations would support the hypothesis that different pathogenic SFG rickettsiae, more or less virulent for humans, coexist in the Catalan region (24). First of all, these geographical discrepancies are similar to those observed in Zimbabwe, where the statement of significant differences in the seroprevalence of SFG rickettsioses in the human population led to the identification of a previously unknown pathogenic rickettsia, *R. africae*, whose geographical distribution overlaps that of *R. conorii*, the MSF agent in Africa (27). Furthermore, studies performed with dogs in the United States showed that depending on the infecting strain (*Rickettsia rickettsii*, *Rickettsia montana*, or *R. rhipicephali*), the serological reactions may be long lasting, transient, or totally absent (9). In Portugal, where *R. conorii* is endemic, long-lasting serological reactions have been observed in dogs (1a). Transient antibody

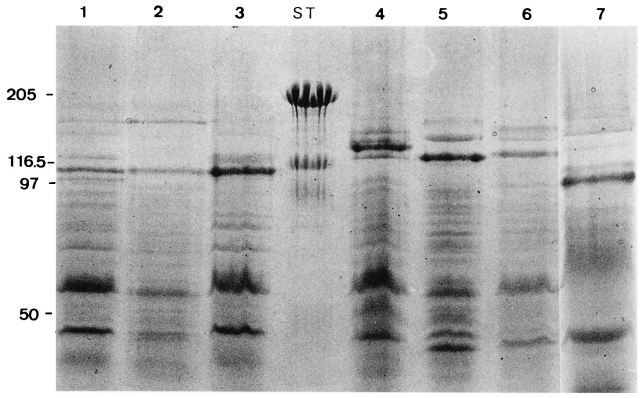

FIG. 5. SDS-PAGE of the antigens of *R. massiliae* (lane 1), *R. rhipicephali* (lane 2), Bar29 (lane 3), *R. conorii* (lane 4), *R. sibirica* (lane 5), *R. slovaca* (lane 6), and *R. helvetica* (lane 7). Standard molecular masses are marked on the left in kilodaltons.

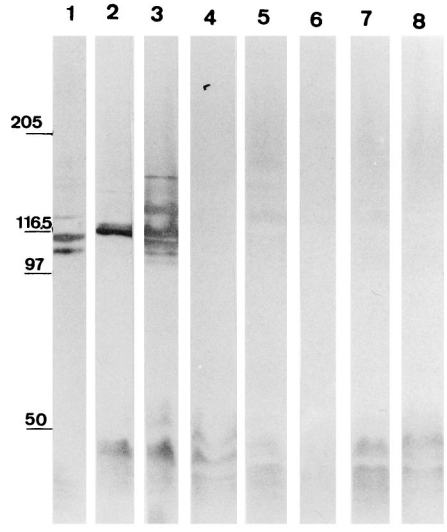

FIG. 6. Western immunoblot assay. Reactions to antibodies of anti-Bar29 sera reactive with *R. rhipicephali* (lane 1), Bar29 (lane 2), *R. massiliae* (lane 3), *R. helvetica* (lane 4), *R. sibirica* (lane 5), Israeli spotted fever rickettsia (lane 6), *R. conorii* (lane 7) and *R. slovaca* (lane 8) are shown. Standard molecular masses are marked on the left in kilodaltons.

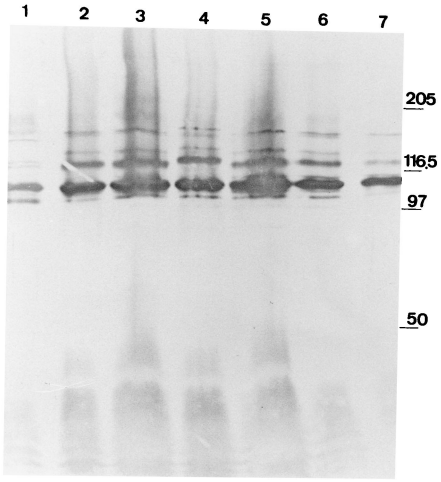

FIG. 7. Western immunoblot assay. Reactions to antibodies of anti-Bar29 sera reactive with *R. massiliae* (lane 1), Bar29 (lane 2), Bar31 (lane 3), Tar1 (lane 4), Tar2 (lane 5), Tar3 (lane 6), and Mtu5 (lane 7) are shown. Standard molecular masses are marked on the right in kilodaltons.

production in Catalan dogs would suggest that the infecting agent is different from *R. conorii*. A third possibility is that different rickettsial strains could be involved in the epidemiology of MSF in Catalonia. In vitro assays showed *R. conorii* and other SFG rickettsiae to be highly susceptible to rifampin (MIC, 0.25 μg/ml) (36). Although these data have not been confirmed by scientific evaluation, practitioners in southern France have often successfully used rifampin in treating children with MSF (35a). In a prospective study, rifampin has, however, been shown to be inefficient in treating children with MSF in Catalonia (8). Our microplaque assays showed that Bar29 is not susceptible to high doses of rifampin (2 μg/ml) in vitro.

These considerations suggest that the possible pathogenicity of Bar29 should be investigated. The pathogenicity of a tick isolate cannot be predicted, as long as the same strain is not either isolated from or inoculated into human beings. SFG rickettsiae have been traditionally classified into two groups:

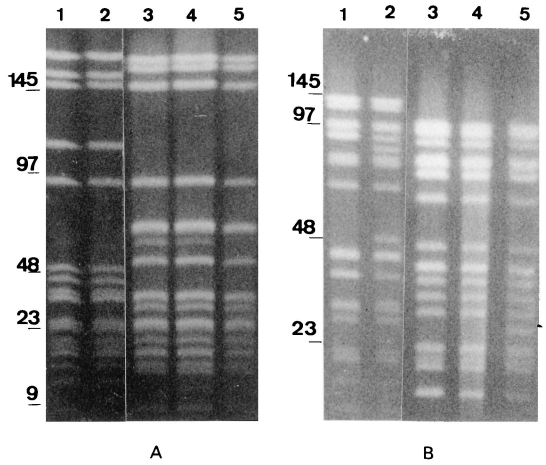

FIG. 8. Ethidium bromide-stained PFGE profiles of the "GS" Greek strain (lane 1), *R. massiliae* (lane 2), Bar29 (lane 3), Bar31 (lane 4), and Tar1 (lane 5) digested with *Sma*I (A) and *Eag*I (B). Standard molecular sizes are marked on the left in kilobases.

pathogenic, when isolated from humans, and nonpathogenic, when isolated from ticks. The term "rickettsiae of unknown pathogenicity" would, however, be more appropriate when referring to tick isolates. This is supported, for example, by the fact that *R. africae* (27) has been considered to be a nonpathogenic rickettsia for about 20 years, as were, previously, *Legionella pneumophila* (47) and *Coxiella burnetii* (11).

Our results show that SFG rickettsiae, other than *R. conorii*, can be found in Catalan *R. sanguineus* ticks. Further investigations with larger tick samples are needed in order to confirm the presence of *R. conorii* in this area. Phenotypic as well as genotypic analysis of human isolates would be of great interest in order to compare them with the tick isolates. Adsorption of sera from patients with MSF to both the *R. conorii* and the Bar29 antigens might be helpful in determining the possible pathogenic role of Bar29 for humans.

ACKNOWLEDGMENTS

We thank Patrick J. Kelly for reviewing the English of the manuscript and Guy Vestris for technical help.

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