JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1993, p. 2625–2633
0095-1137/93/102625-09$02.00/0

Vol. 31, No. 10

Proteinic and Genomic Identification of Spotted Fever Group Rickettsiae Isolated in the Former USSR

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Received 21 January 1993/Returned for modification 29 March 1993/Accepted 6 July 1993

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), restriction fragment length polymorphism of polymerase chain reaction-amplified genes (RFLP-PCR), and pulsed-field gel electrophoresis (PFGE) were used to identify 25 isolates of spotted fever group rickettsiae collected in the former USSR. Six *Rickettsia akari* isolates which were identical to the MK reference strain from the American Type Culture Collection were found. Also, 14 isolates were found to be *Rickettsia sibirica* and identical to reference strain 246. Two of three isolates previously considered as atypical, low-pathogenic strains of *R. sibirica*, were found to be strains of *Rickettsia slovaca*. The third, strain S, was similar in its RFLP-PCR profile to "*R. africae*" sp. nov. (proposed name for a rickettsia pathogenic for human beings in southern Africa) but in its SDS-PAGE and PFGE profiles was unique among spotted fever group rickettsiae. Strain M-1 was confirmed as a genetic variant of *Rickettsia conorii*. The Astrachan isolate, the causative agent of a tick-bite rickettsiosis at the North of the Caspian Sea, showed a previously described RFLP-PCR profile identical to that of the Israeli tick typhus rickettsia, but its SDS-PAGE and PFGE profiles different from those of the other strains tested.

The genus *Rickettsia* is composed of strict intracellular gram-negative bacteria associated with arthropods. Three subgroups are currently described: the typhus group, scrub typhus group, and spotted fever group (SFG) (47).

In the former USSR, three tick- and mite-borne rickettsial diseases have been classically described: Sibirian tick spotted fever typhus caused by *Rickettsia sibirica*, Mediterranean spotted fever caused by *Rickettsia conorii*, and rickettsial pox caused by *Rickettsia akari* (36). Sibirian tick spotted fever typhus (or tick-borne typhus of northern Asia) is the most studied rickettsiosis in the former USSR. Natural foci of this disease extend from the Far East and Siberia through a wide area of central and western Siberia to the Altai region and to the Kazakhstan. Outside of this area of endemicity, rickettsial strains, antigenically indistinguishable from *R. sibirica*, have been isolated in Turkmenistan, Kirghizstan, Armenia, Azerbaijan, Lithuania, the Tula region of Russia, and Slovakia. In this areas, *R. sibirica*-like strains have been isolated from ticks, but no reports of disease among humans have been made. In addition to the above, in Slovakia, Armenia, and Crimea, Ukraine, low-pathogenic strains of SFG rickettsiae were isolated from *Dermacentor marginatus* (36, 41, 44), and these strains differed antigenically and genetically from *R. sibirica*, *R. conorii*, and *R. akari* (4, 35, 43, 44). One of these *Dermacentor* isolates from Slovakia was characterized as a new SFG rickettsial species and named *Rickettsia slovaca* (43). Mediterranean spotted fever in the Black Sea area and rickettsial pox in southern Ukraine were described during outbreaks in 1940 to 1950, but only sporadic cases are recorded now (36).

Recently, a new focus of spotted fever was described in Astrachan on the Caspian Sea (5, 40), and the disease was reported to be caused by an agent similar to that which caused spotted fever in Israel (13). Thus, four SFG rickettsiae have been identified in the former USSR: *R. conorii*, *R. sibirica*, *R. akari* and the agent of Astrachan fever. Many of these isolates were identified by complement fixation and in animal models (4, 36, 41, 44). The purpose of our experiments was to identify and characterize SFG rickettsiae isolated in the former USSR by using modern tools including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), restriction fragment length polymorphism of polymerase chain reaction-amplified genes (RFLP-PCR), and pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

All tested rickettsial strains and their original sources are listed in Table 1. The strains studied were obtained from the collection of the N. F. Gamaleya Research Institute of Epidemiology and Microbiology (Moscow, Russia) and were kept lyophilized at −20°C after isolation and characterization. Lyophilized strains were used for Vero cell monolayer infection. Infected Vero cell monolayers were grown in minimal essential medium (Seromed, Berlin, Germany) supplemented with 4% fetal bovine sera (Seromed) and 1% L-glutamine in a 5% CO_2 atmosphere at 32°C. Five to seven days after inoculation, the infected cells were Gimenez stained (19) and harvested.

As for PCR samples, 1-ml aliquots of highly infected Vero cells were pelleted three times in distilled water by centrifugation at 17,500 × *g* for 5 min and then boiled for 10 min. PCR amplification was performed by using oligonucleotide primer pairs RpCS.877p and RpCS.1258n generated from the citrate synthase gene of *Rickettsia prowazekii* (49), Rr190.70p and Rr190.602n generated from the 190-kDa antigen gene of *Rickettsia rickettsii* (3), and BG1-21 and BG2-20

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TABLE 1. Rickettsial strains studied

Species and strain or description	Origin	Location and year of isolation	Investigator(s) or reference	Source[a]
R. sibirica				
Nezvetaev (232)	Human	Altai, Russia, 1946	Kulagin and Korschunova (10)	G. Dasch (Naval Medical Research Institute, Bethesda, Md.)
K-1 (246)	*Dermacentor nuttalli*	Krasnojarsk, Russia, 1949	Golinevitch (11)	GRIC
Altai 24/86	*Haemophysalis concinna*	Altai, Russia, 1986	Rudakov	GRIC
Altai 81/88	*Dermacentor silvarum*	Altai, Russia, 1988	Rudakov and Samoilenko	GRIC
Armenia-25[b]	*Dermacentor marginatus*	Armenia, 1972	Bazlikova	GRIC
Baevo 105/87	*Dermacentor marginatus*	Altai, Russia, 1987	Rudakov	GRIC
Baevo 107/87	*Dermacentor marginatus*	Altai, Russia, 1987	Rudakov	GRIC
Burjatia 91/85	*Dermacentor nuttali*	Burjatia, Russia, 1985	Reschetnikova	GRIC
Crimea-108[b]	*Dermacentor marginatus*	Crimea, Ukraine, 1977	Vorontzova (44)	GRIC
Gornyi 54/88	*Dermacentor nuttalli*	Altai, Russia, 1988	Rudakov and Schpynov	GRIC
Irkutsk 9/83	*Dermacentor nuttalli*	Irkutsk, Russia, 1983	Reschetnikova	GRIC
Krasnojarsk 10/91	*Dermacentor nuttalli*	Krasnojarsk, Russia, 1991	Samoilenko	GRIC
Krasnojarsk 15/91	*Dermacentor nuttalli*	Krasnojarsk, Russia, 1991	Samoilenko	GRIC
M	*Dermacentor marginatus*	Armenia, 1954	Kotsinjan	GRIC
Prymorye 6/83	*Dermacentor silvarum*	Prymorye region, Russia, 1983	Reschetnikova	GRIC
Prymorye 20/84	*Haemophysalis concinna*	Prymorye region, Russia, 1984	Reschetnikova	GRIC
S[c]	*Rhipicephalus sanguineus*	Armenia, 1955	Kotsinjan	GRIC
Sidro	Human	Novosibirsk, Russia, 1954	Schaiman	GRIC
Suzun 11/89	*Dermacentor silvarum*	Novosibirsk, Russia, 1989	Rudakov	GRIC
R. slovaca				
13-B	*Dermacentor marginatus*	Slovakia, 1969	Urvolgyi and Brezina (43)	G. Dasch
R. conorii				
Moroccan	Unknown	Morocco	(11)	ATCC VR-613
M-1	*Rhipicephalus sanguineus*	Suchumi, Georgia, 1947	Golinevitch (21)	GRIC
Indian tick typhus rickettsia[e]	*Rhipicephalus sanguineus*	Kashmir, India, 1950	Philip et al. (31)	C. B. Philip, GRIC
Kenyan tick typhus rickettsia	*Haemophysalis leachii*	Nairobi, Kenya, 1953	(11)	G. Dasch
New isolate[d]	Human	Astrachan region, Russia, 1991	Makarova et al.	GRIC
"*R. africae*" sp. nov. ESF 2500-1	*Amblyomma variegatum*	Slulu province, Ethiopia		G. Dasch
IsTT CDC-1[f]	*Rhipicephalus sanguineus*	Israel	Goldwasser et al. (20)	G. Dasch
R. akari				
MK	Human	United States, 1946	Huebner et al. (22)	ATCC VR-148
Toger	Human	Ukraine, 1950	Kulagin	GRIC
Askalunin	Human	Ukraine, 1950	Kulagin	GRIC
CK	Human	Ukraine, 1950	Kulagin	GRIC
As4	*Allodermanyssus sanguineus*	Ukraine, 1950	Kulagin	GRIC
M-3	Domestic mice	Ukraine, 1950	Kulagin	GRIC
R-1	Domestic rat	Ukraine, 1950	Kulagin	GRIC

[a] ATCC, American Type Culture Collection; GRIC, N. F. Gamaleya Research Institute collection, Moscow, Russia.
[b] Atypical strains of *R. sibirica* and were identified as *R. slovaca* per our study.
[c] Atypical strain of *R. sibirica*; species denomination should be reconfirmed.
[d] Species denomination should be determined.
[e] Species should be reconfirmed.
[f] IsTT, name has not been recognized.

(BG1-2) and BG5-23 and BG6-22 (BG5-6), both generated from the 120-kDa antigen gene of *R. rickettsii* (18) (Bioprobe Systems, Montreuil-sous-Bois, France). PCR amplification consisted of 35 cycles (denaturation at 95°C for 20 s, annealing at 48°C for 30 s, and sequence extension at 60°C for 2 min) according to the protocol of Regnery et al. (35). The reaction was performed in a 100-μl volume of the reaction mixture containing 10 μl of the boiled infected cells, 59.5 μl of distilled water, 10 μl of 10× *Taq* buffer (Boehringer Mannheim, Meylan, France), 10 μl of deoxynucleotide triphosphates (2% dATP, 2% dTTP, 2% dCTP, 2% dGTP [Boehringer Mannheim] in distilled water), 5 μl of each component of the primer pair, and 0.5 μl of *Taq* polymerase (5,000 U/ml; Boehringer Mannheim) by using a thermal cycler (PREM III; Lep Scientific, Flobio, Courbevoie, France). To determine that amplification had occurred, 10 μl of the PCR product was electrophoresed in a 1% agarose gel (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 100 V simultaneously with DNA molecular weight marker VI (a mixture of pBR328 DNA digested with restriction endonuclease *Bgl*I and of pBR328 DNA digested with restriction endonuclease *Hin*fI [Boehringer Mannheim]). Aliquots (23.3

μl) of the amplified product were then digested with 1 μl (10 to 20 U) of *Rsa*I, *Pst*I, and *Alu*I endonucleases (New England Biolabs, Inc., Beverly, Mass.) for 2 h at 37°C, and the restriction products were separated in an 8% polyacrylamide gel at 100 V for 4 h, stained in a 0.5% solution of ethidium bromide, and transilluminated (365 nm). DNA molecular weight marker V (plasmid pBR322 *Hae*III-digested DNA; Boehringer Mannheim) was run simultaneously with the samples to determine the molecular weights of the observed DNA fragments.

For SDS-PAGE and PFGE, rickettsiae were purified as follows. Highly infected Vero cells harvested from 10 150-cm^2 flasks were pelleted by centrifugation (5,000 × *g* for 20 min), resuspended in K-36 solution (0.1 M KCl, 0.15 M NaCl, 0.05 M potassium phosphate buffer [pH 7.0] [46]), and sonicated in an ice bath. Host cell debris was separated by two low-speed centrifugations (150 × *g* for 10 min), and the supernatant was overlaid on a 25% sucrose cushion (48) and centrifugated at 12,000 × *g* for 40 min. The resulting pellet was resuspended in 2 ml of K-36 solution, and the mixture was placed on a 28 to 45% linear Renografin (Radioselectan, Schering, France) gradient and centrifugated at 25,000 rpm for 1 h (L3-50 centrifuge, rotor SW-27; Beckman, Fullerton, Calif.). The rickettsial material was collected with a sterile syringe, diluted with K-36 buffer, and pelleted at 12,000 × *g* for 10 min. The last step was repeated, and purified rickettsiae were suspended in 1 ml of K-36 buffer. The protein concentration of the purified rickettsial suspensions was determined by the Peterson modification of the Lowry method in the presence of 5% SDS (30).

PAGE was performed according to the method of Laemmli (25), with Bio-Rad (Richmond, Calif.) reagents and equipment. Briefly, rickettsial protein (10 μg per well) solubilized in sample buffer at room temperature was loaded on a polyacrylamide gel (3% stacking gel and 7.5% separating gel), electrophoresed at 30 mA, and stained with Coomassie R-250. High molecular-weight-range prestained SDS-PAGE standards, containing myosin (molecular mass, 205 kDa), β-galactosidase (116.5 kDa), bovine serum albumin (80 kDa), and ovalbumin (49.5 kDa) (Bio-Rad), were used as markers.

For PFGE, chloramphenicol (final concentration, 25 μg/ml; Merck Sharp & Dohme-Chibert, Chibert Div., Paris, France) was added to an infected Vero cell culture 24 h before harvest to allow DNA replication to reach completion (24). The suspension of purified rickettsiae was adjusted to densities equal to an optical density of 1.2 at 260 nm and of 0.8 at 280 nm in K-36 buffer and mixed with 1 volume of the 1% InCert low-melting-point agarose (FMC Bioproducts, Rockland, Maine) at 42°C. The suspension was then quickly transferred to 100-μl sample holders and allowed to cool on ice for 5 min. Solidified plugs (blocks) of agarose were transferred to a lysis mixture containing 3 to 5 volumes of ESP buffer (0.5 M EDTA [pH 8.0], 1% SDS, 1 mg of proteinase K [Boehringer Mannheim] per ml) and incubated at 50°C for 24 h. The blocks were transferred in ESP buffer, and incubation was repeated under the same conditions. The blocks were washed twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]) for 30 min at room temperature and then incubated twice, 1 h each, at 50°C in TE buffer supplemented with 0.04 mg of phenylmethylsulfonyl fluoride (Sigma) per ml to inactivate proteinase K. After two more washes in TE buffer at room temperature (by using 5 volumes of buffer to 1 volume of gel plugs), the blocks were stored in ES (0.5 M EDTA [pH 8.0], 1% SDS) buffer at 4°C.

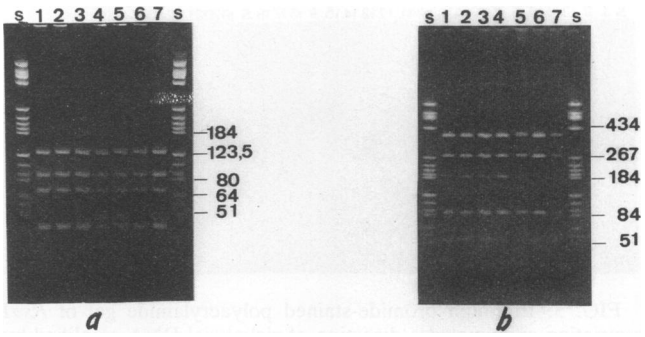


FIG. 1. RFLP-PCR analysis for *R. akari* strains. DNA was amplified by using RpCS.877-RpCS.1258pn (A) and BG5-6 (B) primer pairs and digested by *Alu*I and *Rsa*I restriction endonucleases, respectively. Lanes: 1, strain MK; 2, strain Toger; 3, strain As4; 4, strain R-1; 5, strain M-3; 6, strain Askalunin; 7, strain CK; s, DNA size marker V (with sizes [in base pairs] indicated on the right).

Restriction endonucleases *Sma*I (CCCGGG), *Eag*I (CGG CCG), and *Bss*HII (GCGCGC) (Boehringer Mannheim) were used for DNA digestion (38). Before digestion, agarose blocks were rinsed twice for 15 min with TE buffer. Digestion was done with 30 U of enzyme at 25°C for *Sma*I, at 37°C for *Eag*I, and at 50°C for *Bss*HII for 2 h and repeated three times. Digestion was stopped by replacing the restriction mixture with 1 ml of ES buffer for 2 h of incubation at 50°C.

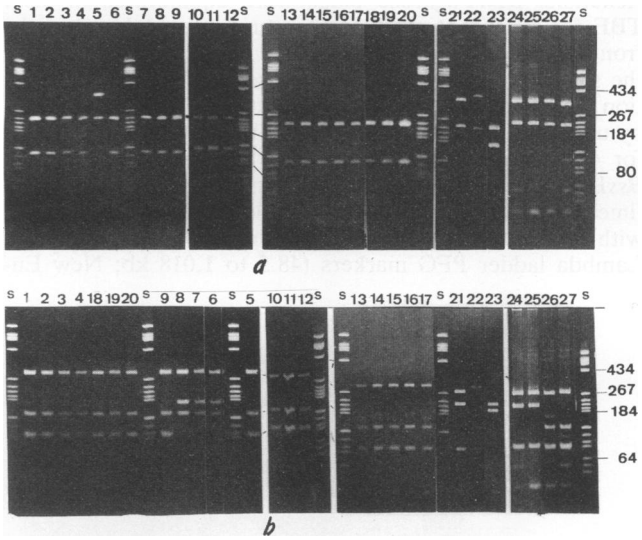


FIG. 2. Ethidium bromide-stained polyacrylamide gel of *Rsa*I (a) and *Pst*I (b) restriction endonuclease digestion of rickettsial DNA amplified by Rr190.70-602pn primer pairs. Lanes: 1, *R. sibirica* M; 2, *R. sibirica* Prymorye 6/83; 3, *R. sibirica* Sidro; 4, *R. sibirica* Baevo 107/87; 5, strain S; 6, strain Armenia-25; 7, *R. slovaca* 13-B; 8, strain Crimea-108; 9, *R. sibirica* 232; 10, *R. sibirica* 246; 11, *R. sibirica* Gornyi 54/88; 12, *R. sibirica* Prymorye 20/84; 13, *R. sibirica* Altai 24/86; 14, *R. sibirica* Suzun; 15, *R. sibirica* Irkutsk 9/83; 16, *R. sibirica* Baevo 105/87; 17, *R. sibirica* Burjatia 91/85; 18, *R. sibirica* Altai 10/88; 19, *R. sibirica* Krasnojarsk 10/91; 20, *R. sibirica* Krasnojarsk 15/91; 21, *R. conorii* Moroccan; 22, Astrachan isolate; 23, *R. conorii* M-1; 24, *R. conorii* Indian tick typhus strain; 25, *R. conorii* Kenyan tick typhus strain; 26, IsTT 27, "*R. africae*" sp. nov, ESF 2500-1; s, DNA size marker V (with sizes [in base pairs] indicated on the right).

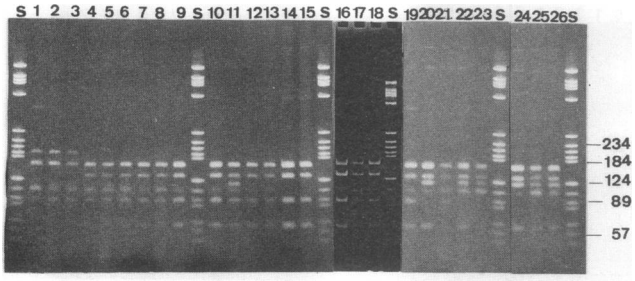


FIG. 3. Ethidium bromide-stained polyacrylamide gel of *Rsa*I restriction endonuclease digestion of rickettsial DNA amplified by BG1-2 pair primers. Lanes: 1, *R. slovaca* 13-B; 2, Crimea-108; 3, Armenia-25; 4, *R. sibirica* 232; 5, *R. sibirica* Prymorye 6/83; 6, *R. sibirica* Baevo 107/87; 7, *R. sibirica* M; 8, *R. sibirica* Sidro; 9, *R. sibirica* Altai 24/86; 10, *R. sibirica* Suzun; 11, *R. sibirica* Irkutsk 9/83; 12, *R. sibirica* Baevo 105/87; 13, *R. sibirica* Burjatia 91/85; 14, *R. sibirica* Krasnojarsk 10/91; 15, *R. sibirica* Krasnojarsk 15/91; 16, *R. sibirica* 246; 17, *R. sibirica* Gornyi 54/88; 18, *R. sibirica* Prymorye 20/84; 19, *R. africae* sp. nov., ESF 2500-1; 20, *R. conorii* Moroccan; 21, strain S; 22, Astrachan isolate; 23, IsTT; 24, *R. conorii* M-1; 25, *R. conorii* Kenyan tick typhus strain; 26, *R. conorii* Indian tick typhus strain; s, DNA size markers (with sizes [in base pairs] indicated on the right).

ES buffer was decanted, and 1 ml of ESP buffer was added. The plugs were then ready for electrophoresis.

The CHEF DR II (Bio-Rad) PFGE system was used with a 1% agarose (Sigma) running gel in 0.5× TBE buffer (0.0455 M Tris-borate, 0.001 M EDTA [pH 8.0]). The digested rickettsial DNA-agarose plugs were equilibrated in 0.5× TBE for 30 min at room temperature twice, placed in the front of the well, and overlaid with liquid agarose by using the same running buffer and agarose concentration. Migration was done in 0.5× TBE buffer at 14°C for 24 h at 200 V by using a pulse time of 3 to 10 s for *Sma*I-digested blocks, for 48 h at 150 V at ramp pulse time of 5 to 120 s for *Bss*HII-digested blocks, and for 33 h at 180 V at ramp pulse time of 5 to 20 s for *Eag*I-digested blocks. Gels were stained with ethidium bromide after completion of migration. Lambda ladder PFG markers (48.5 to 1,018 kb; New England Biolabs, Inc.) were used as molecular weight standards.

Molecular weight estimation and profile comparison. All gels for SDS-PAGE, RFLP-PCR, and PFGE were computerized by using the Imager documentation system (Appligene SA, Illkirch, France). The molecular weights of the proteins and the sizes of the DNA bands were determined according to their relative electrophoretic mobility in comparison with correspondent standards by using the QGel-DTM program (Quantigel Corporation, Madison, Wis.). A data bank created in the laboratory by using this system allowed us to compare new data with previously obtained results for rickettsiae, including "*R. africae*" sp. nov. (proposed name for the pathogenic strain identical to Ethiopian tick typhus rickettsiae [23]), *R. akari*, *R. australis*, *R. bellii*, *R. conorii*, *R. helvetica*, *R. japonica*, *R. massiliae*, *R. montana*, *R. parkeri*, *R. rhipicephali*, *R. rickettsii*, *R. sibirica*, *R. slovaca*, Israeli tick typhus rickettsiae (IsTT), and Thai tick typhus rickettsiae (14, 39).

RESULTS

PCR amplification and RFLP analysis. The results of the RFLP-PCR analysis are shown in Fig. 1 to 3. Amplification with the citrate synthase gene primer pair following *Alu*I digestion was used as a first step. For all SFG rickettsial strains studied with the exception of *R. akari* strains, *Alu*I digestion profiles were identical and consisted of four bands of 131, 100, 96, and 47 bp, common to other SFG rickettsiae. Six newly investigated strains of *R. akari* isolated from different sources showed an *Alu*I profile identical to that of reference strain *R. akari* MK. This profile was found to be specific for this species and consisted of four bands of 126, 85, 69, and 48 bp (Fig. 1a).

By using the Rr190.70p and Rr190.602n primer pair, all rickettsial strains studied except *R. akari* were amplified (Fig. 2). For 17 isolates previously identified as *R. sibirica*, 14 had the same *Pst*I and *Rsa*I digestion profiles of the amplified DNA product as the standard prototype strain 246 (lane 10) and neotype strain 232 (lane 9): after *Pst*I digestion three bands of 311, 139, and 90 bp and after *Rsa*I digestion two bands of 234 bp consisting of two comigrating fragments

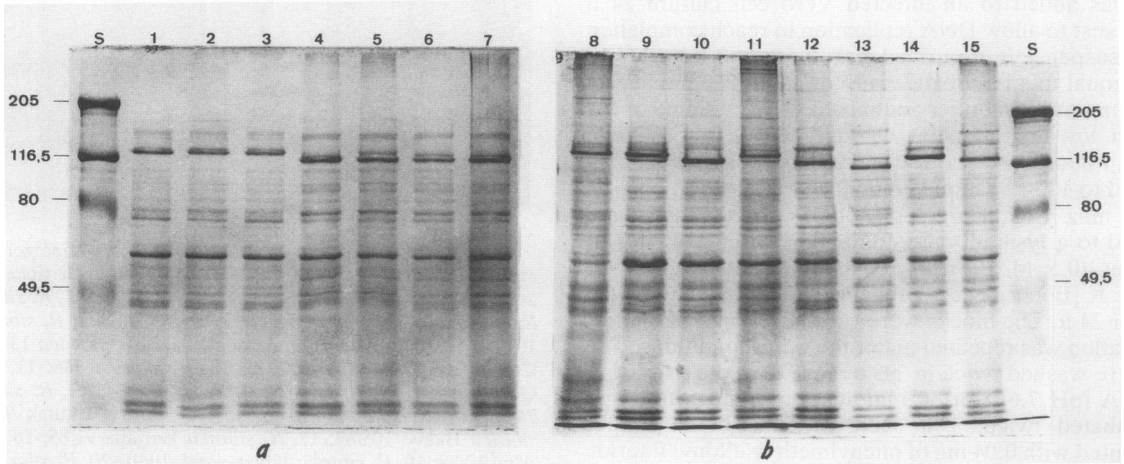
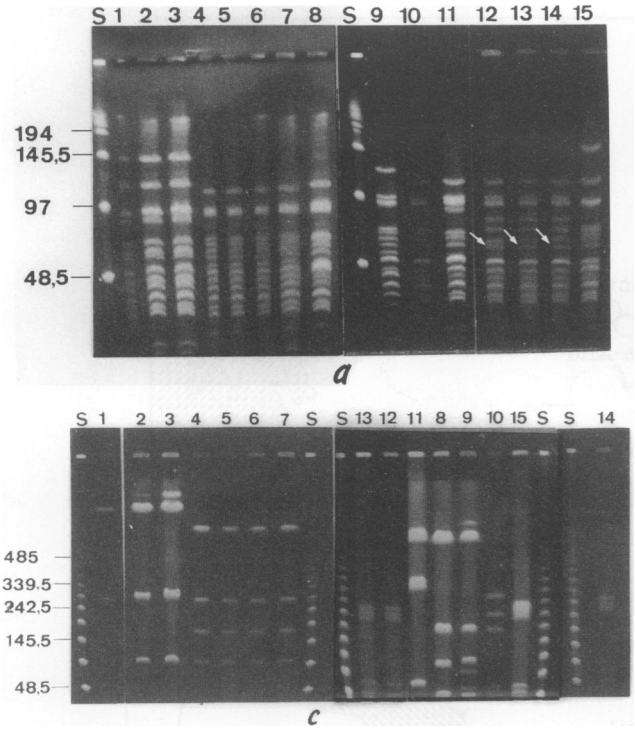


FIG. 4. Coomassie R-250-stained SDS-PAGE profiles of *R. sibirica* and *R. sibirica*-like strains (a) and *R. conorii* complex strains (b). Lanes: 1, *R. slovaca* 13-B; 2, strain Armenia-25; 3, strain Crimea-108; 4, *R. sibirica* 246; 5, *R. sibirica* Gornyi 54/88; 6, *R. sibirica* Prymorye 20/84; 7, *R. sibirica* 232; 8, *R. conorii* Moroccan; 9, *R. conorii* M-1; 10, *R. conorii* Indian tick typhus strain; 11, *R. conorii* Kenyan tick typhus strain; 12, "*R. africae*" sp. nov., ESF 2500-1; 13, S; 14, Astrachan isolate; 15, IsTT; s, standard proteins, with molecular masses (in kilodaltons) indicated on the left and right.



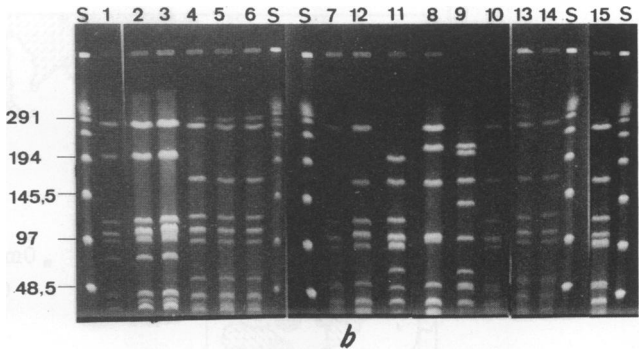


FIG. 5. *Sma*I (a)-, *Eag*I (b)-, and *Bss*HII (c)-digested profiles of the chromosomal DNA (stained with ethidium bromide) of strains studied after PFGE migration. Lanes: 1, *R. slovaca* 13-B; 2, strain Armenia-25; 3, strain Crimea-108; 4, *R. sibirica* 246; 5, *R. sibirica* Gornyi 54/88; 6, *R. sibirica* Prymorye 20/84; 7, *R. sibirica* 232; 8, strain S; 9, "*R. africae*" sp. nov., ESF 2500-1; 10, IsTT; 11, Astrachan isolate; 12, *R. conorii* Moroccan; 13, *R. conorii* M-1; 14, *R. conorii* Kenyan tick typhus strain; 15, *R. conorii* Indian tick typhus strain; s; lambda ladder PFG marker (fragment sizes [in kilobases] indicated on the left). Arrows indicate the distinguishable comigrating DNA bands in *R. conorii* Moroccan and M-1 and the Kenyan tick typhus strain.

and one band of 110 bp. Three strains, Crimea-108 (lane 8), isolated in Crimea, and Armenia-25 (lane 6) and S (lane 5), both isolated in Armenia, differed from *R. sibirica*. The strains Crimea-108 and Armenia-25 were identical. Their *Rsa*I digestion profiles were identical to those of *R. sibirica* and *R. slovaca* (lane 7), but their *Pst*I profile was identical to that of *R. slovaca* and thus differed from *R. sibirica* by lacking a 90-bp band. For strain S, the *Rsa*I and *Pst*I profiles were similar to those of "*R. africae*" sp. nov. and *R. sibirica*, respectively. The Astrachan isolate digestion profiles (lane 22) were identical to that of IsTT (lane 24). Strain M-1 (lane 23) differed from other SFG rickettsiae in the size of the 387- to 390-bp amplified DNA fragment, which was smaller and digested in two bands by both *Rsa*I (217 and 170 bp) and *Pst*I (202 and 188 bp).

By using primer pairs generated from the 120-kDa antigen gene of *R. rickettsii*, all strains studied including *R. akari*, were amplified. BG1-2 primer pairs amplified DNA of all *R. sibirica* strains and after *Rsa*I digestion revealed five bands of 165, 132, 104, 86, and 62 bp (Fig. 3, lanes 4 to 18). Strain S (lane 21) and "*R. africae*" sp. nov. (lane 19) had profiles identical to that of *R. sibirica*. Strains Crimea-108 (lane 2) and Armenia-25 (lane 3) after *Rsa*I digestion exhibited only four bands of 190, 164, 103, and 89 bp identical to those of *R. slovaca* (lane 1); the Astrachan human isolate (lane 22) after *Rsa*I digestion exhibited five bands of 165, 130, 118, 104, and 62 bp identical to those of IsTT (lane 23) and *R. conorii* Moroccan (lane 20) and M-1 (lane 24). BG5-6-amplified fragment DNA of the six *R. akari* strains studied were identical to that of reference strain MK and exhibited four bands of 395, 288, 89, and 56 bp (Fig. 1b).

SDS-PAGE analysis. SDS-PAGE analysis (Fig. 4) showed that all strains studied had very similar protein profiles, especially in the molecular mass range lower than 60 to 62 kDa. The main differences were found in the high-molecular-weight proteins. Four *R. sibirica* strains, 246, 232, Gornyi 54/88, and Prymorye 20/84, exhibited four high-molecular-mass proteins with masses of 114, 121, 152, and 183 kDa (lanes 4 to 7). Strains Crimea-108 (lane 3) and Armenia-25 (lane 2), being similar, had four bands of 121, 126, 146, and 152 kDa which were identical to those of *R. slovaca* (lane 1). Strain S exhibited four protein bands of 110, 127, 150, and 170 kDa (lane 13). This profile was specific and differed from those of *R. slovaca*, *R. sibirica*, *R. conorii*, "*R. africae*" sp. nov., and IsTT. Moroccan, M-1, and the Kenyan tick typhus strain of *R. conorii* had three bands of 127, 139, and 145 kDa (lanes 8, 9, and 11, respectively). Only the Indian tick typhus strain differed markedly from other strains studied, including *R. conorii*, and had two proteins, 122 and 145 kDa (lane 10). As for the Astrachan isolate protein profile, the mass of the main high-molecular-mass protein was 120 to 122 kDa, and four minor proteins with masses of 130, 139, 150, and 163 kDa were also found (lane 14). This protein profile was unique and differed from those of *R. conorii* (lane 8) and IsTT (lane 15).

PFGE. PFGE profiles were compared after digestion by the low-frequency restriction endonucleases *Sma*I, *Eag*I, and *Bss*HII. The migration profiles obtained are shown in Fig. 5. It was found that strains Armenia-25 (lane 2) and Crimea-108 (lane 3) were identical to *R. slovaca* (lane 1). *R. sibirica* Gornyi 54/88 (lane 5) and Prymorye 20/84 (lane 6) were identical and indistinguishable from standard strains 246 and 232 (lanes 4 and 7). *R. conorii* Moroccan and M-1, and the Kenyan tick typhus strain had common *Bss*HII and *Eag*I digestion profiles (lanes 12 to 14, respectively). These three strains revealed subtle reproducible differences in the relative electrophoretic mobility of two *Sma*I restriction fragments with lengths of 60 to 65 bp. Only the Indian tick typhus strain had a very specific profile, which differed from those of the other strains studied (lane 15).

PFGE profiles obtained after *Sma*I, *Eag*I, and *Bss*HII digestion were specific for strain S (lane 8) and the Astrachan isolate (lane 11); they differed in the range and number of bands between each other and from those of *R. sibirica*, *R. slovaca*, *R. conorii*, "*R. africae*" sp. nov., and IsTT.

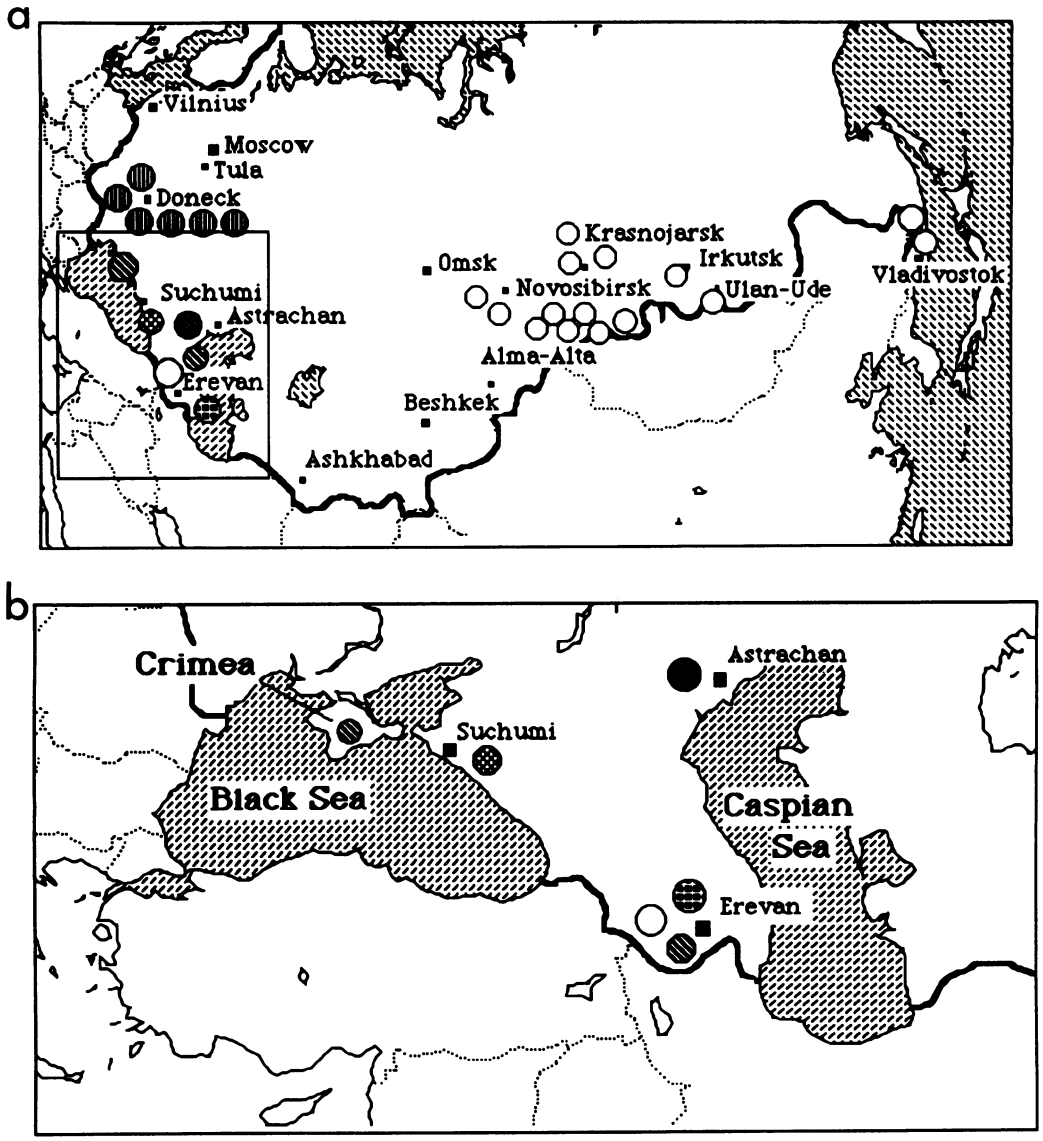


FIG. 6. Geographical distribution of the SFG rickettsiae strains studied in the territory of the former USSR (a) and in the Black Sea and Caspian Sea areas (b). ○, *R. sibirica*; ◍, S strain; ◍, *R. slovaca*, ●, Astrachan strain; ◍, *R. conorii*; ◍, *R. akari*.

DISCUSSION

At the present time, SFG rickettsiae include eleven different species (10, 42, 43, 47) and three isolates which are not recognized currently as species but differ from the others: IsTT (20), "*R. africae*" sp. nov. (16, 33), and the Thai tick typhus rickettsia (37). In addition, a number of new pathogenic and nonpathogenic rickettsiae isolated from ticks and patients have been described recently (5, 7, 9, 13, 23, 40, 50).

SFG rickettsiae currently defined have been identified serologically. Roughly, the species definition for the SFG rickettsiae is a serotype. It was first determined by complement fixation (34), later by using the toxin neutralization test in mice (11) and cross-immunization in guinea pigs (12), and finally by cross-immunofluorescence with mouse sera (32). These techniques have been of value but are not highly reproducible and are difficult to carry out because of the fact that no data base can be created and, therefore, a new isolate must be compared with all previously described species. A bank of polyclonal sera is also needed to conduct these studies. Monoclonal antibodies are useful in differentiating strains (2, 15), but, so far, there is no available panel of monoclonal antibodies to identify isolates. SDS-PAGE analysis is helpful in the identification of *Rickettsia* isolates (7, 23, 29, 50). Data could be recorded and compared without the necessity of running all species each time, thanks to computerized programs. However, the proteins of interest are the outer membrane proteins (190 and 120 kDa) which determine the serotype of SFG rickettsiae (1, 2). Within the same species, variations in the molecular weights of these proteins have been shown, such as in *R. rickettsii* (17). Recently RFLP-PCR was introduced as a tool for classification and identification of *Rickettsia* spp. (35). Regnery et al. suggested the use of two primer pairs, one obtained from the citrate synthase gene, which allows recognition of SFG rickettsiae, and a 190-kDa antigen gene-derived primer pair, which allows identification of SFG rickettsiae at the species

level (35). Recently, the 120-kDa antigen gene-derived primer pairs were introduced in order to differentiate species and strains, such as IsTT and "*R. africae*" sp. nov., that were not fully identified by the previously described methods (14, 26). The use of PFGE for classification and identification of rickettsiae has been recently described (39). We believe that it is of value in studying the total genome in spite of the availability of limited pieces of genes.

In the present work we decided to study the isolates obtained in the former USSR by using SDS-PAGE protein analysis, RFLP-PCR, and PFGE and to compare them with the isolates in our data bank provided by previous or parallel work done in the laboratory. The isolates studied were identified previously by serological methods. These three methods gave identical results for the determination of the strain, with the exceptions of the Astrachan isolate and strain S. The former showed an RFLP-PCR profile identical to that of IsTT and therefore was considered similar to it, which is in accordance with the clinical and epidemiological data for the two diseases caused by these two rickettsiae (13, 40). In the present work, we report that these two rickettsial isolates differ in their protein and PFGE profiles. Strain S, being identical to "*R. africae*" by RFLP-PCR, differed from it when using the two other methods. These facts confirm the limits of RFLP-PCR analysis for identifying SFG rickettsiae that we had previously found when comparing *R. parkeri* and "*R. africae*" sp. nov. (14). As for other strains, because the data are concordant we believe that we can correctly classify the isolates found in the former USSR. Finally, comparing the three methods, we believe that RFLP-PCR is the easiest and most rapid method for the identification of rickettsiae. As previously shown, when RFLP-PCR provides an identification that is in accordance with known epidemiological data, this method is probably sufficient for identification (7). Confirmation by SDS-PAGE is necessary in cases of questionable results. PFGE also allows a precise identification and classification.

Fourteen strains of *R. sibirica* isolated from foci of endemicity were shown to be the same with the identification tools used. Three strains were considered to be atypic, low-pathogenic *R. sibirica* at the moment of isolation. In fact, Crimea-108 and Armenia-25 isolated outside of the foci of endemicity were identical and indistinguishable from *R. slovaca*. Our data support the fact that they are *R. slovaca* isolates. *R. slovaca* is a widely distributed species, described first in Czechoslovakia (43) and subsequently recognized in France, Switzerland (6, 8), and Yugoslavia (27). Moreover, all the *R. slovaca* isolates were obtained from *D. marginatus* ticks. The pathogenic role of this rickettsia is unknown, but Czechoslovakian investigators suspected its role in meningoencephalitis in humans (28).

Strain S, sharing common RFLP-PCR profiles with "*R. africae*," whose pathogenic role has been described recently (23), differed from it in protein and chromosomal DNA digestion profiles. It has been isolated in Armenia in the region between two natural foci of the "fievre boutonneuse" in the former USSR (36), where both *R. slovaca* and *R. sibirica* are prevalent; strain S needs further identification. Finally, the presence of *R. conorii* in the Black Sea basin was confirmed. Strain M-1 (21), isolated from a tick, differed from previously described *R. conorii* strains but was identical to the Barbash strain (14), which has been recently reclassified from *R. sibirica* to *R. conorii* (45). The Barbash strain isolated in the Far East of Russia was first considered to be a reference *R. sibirica* strain. Obviously, that was a mistake, but it is now difficult to distinguish whether this reclassification is the result of a misidentification at the time of identification or a later mixing of the strains. We will not conclude between these two hypotheses because the data are not sufficient to extend so widely the distribution area of *R. conorii*.

The data presented in this work extend the knowledge of the geographic distribution of SFG rickettsiae (Fig. 6). In fact, *R. sibirica* is prevalent in the Asian part of Russia and extends to Armenia and China (15, 50), and the isolates are very homogeneous. *R. conorii* is prevalent in the Black Sea basin. The *R. akari* isolates from southern Ukraine are very homogeneous in this study. The Astrachan isolate, which is pathogenic in humans in the Caspian Sea area, differs slightly from IsTT, to which it was previously thought to be identical (13). The extent of divergence of this isolate from other SFG rickettsiae remains to be determined and may eventually lead to a proposal of species denomination. Our results also show that the distribution of *R. slovaca* is further east than was first described. In addition, a new SFG rickettsial isolate, strain S, was found in Armenia. In conclusion, four pathogenic SFG rickettsiae are present in the territory of the former USSR, and the pathogenic roles of *R. slovaca* and strain S remain to be determined.

ACKNOWLEDGMENTS

We thank P. Kelly for the review of the manuscript and I. Domingo and V. Pinero for secretarial assistance.

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