GENOMIC IDENTIFICATION OF *RICKETTSIA SLOVACA* AMONG SPOTTED FEVER GROUP RICKETTSIA ISOLATES FROM *DERMACENTOR MARGINATUS* IN ARMENIA

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Summary. – Restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified genes was used for genomic identification of Armenian isolates of the Spotted fever group (SFG) rickettsiae with unclear taxonomic position. Analysis was performed by using one genus-specific primer pair derived from *R. prowazekii* citrate synthase gene and two species-specific primer pairs derived from *R. rickettsii* genes for 190 K and 120 K antigens following *Alu*I, *Pst*I and *Rsa*I digestion of amplicons. All tested rickettsial SFG Armenian isolates from *Dermacentor marginatus* were identified as *R. slovaca*. The geographic distribution and genetic homogeneity of *R. slovaca* strains are discussed.

Key words: Spotted fever group rickettsiae; *R. slovaca*; Armenian isolates; genotype PCR/RFLP analysis; *D. marginatus*

Introduction

A new type of SFG rickettsiae has been established in Slovakia in 1968 (Brezina *et al.*, 1968). In this area strains with antigenic properties similar to SFG rickettsiae but different from them by their low virulence for experimental animals were isolated from *D. marginatus* ticks. They were classified as a new species and named *R. slovaca* (Urvölgyi and Brezina, 1978). Soon after, a number of strains closely related to *R. slovaca* were isolated in Southern Germany, Hungary, Austria, Armenia, and Slovakia as well (Řeháček and Tarasevich, 1968). Most of them have been considered as *R. slovaca*, but the isolates from Armenia and several isolates from Slovakia as *R. sibirica* sero-variants (Tarasevich *et al.*, 1976; Makarova *et al.*, 1978; Řeháček and Tarasevich, 1988).

Currently the molecular genetic methods based on RFLP analysis of the fragments of the whole chromosomal DNA allow to determine the taxonomic position of SFG rickettsial isolates. By RFLP analysis after PCR amplification of part of genes encoding the 190 K (Regnery *et al.*, 1991) and 120 K (Eremeeva *et al.*, 1993, 1994) *R. rickettsii* outer membrane protein antigens, *R. slovaca* strain 13-B isolated in central Slovakia from *D. marginatus* was considered to have distant position from other SFG rickettsiae. This was confirmed by pulsed field gel electrophoresis (PFGE) analysis of chromosomal DNA (Roux and Raoult, 1993; Eremeeva *et al.*, 1993), Southern blot hybridization analysis with DNA probes generated from *R. rickettsii* (Ralph *et al.*, 1985), and SDS-PAGE immunoblot analysis (Eremeeva *et al.*, 1993; Beati *et al.*, 1993). Recently similar approach allowed to identify *R. slovaca* in France (Beati *et al.*, 1993), Switzerland (Beati *et al.*, 1992) and Yugoslavia (Manor *et al.*, 1992).

The data of the genotype identification of atypical SFG rickettsial strains isolated from *D. marginatus* in Armenia and kept in the collection of the Gamaleya Research Institute of Epidemiology and Microbiology are presented in this paper.

Materials and Methods

Rickettsiae. The tested isolates and their origin are listed in Table 1. *R. slovaca* strain 13-B and *R. sibirica* strain K-1 (246) were used as standard strains.

Table 1. Species and strains of rickettsiae studied

Species and strain	Origin	Place and year of isolation	Reference	Note
R. slovaca 13-B	*D. marginatus*	Slovakia 1969	Urvölgyi and Brezina (1978)	G. Dasch, Naval Medical Research Institute
R. slovaca[a] Crimea-108	*D. marginatus*	Crimea Ukraine 1977	Vorontzova (1980)	RGRICR[b]
R. slovaca[a,c] Armenia 25	*D. marginatus*	Armenia 1972	Bázliková[c]	RGRIC
R. sibirica serovariant Armenia 74	D. marginatus	Armenia 1974	Makarova, Tarasevich, Plotnikova (1978)	RGRIC
R. sibirica[c] atypical strain Armenia 29	*D. marginatus*	Armenia 1972	Bázliková[c]	RGRIC
R. sibirica[c] atypical strain Armenia 5	*D. marginatus*	Armenia 1972	Bázliková[c]	RGRIC
R. sibirica K-1 (246)	D. nuttalli	Russia Krasnojarsk 1949	Golinevich	RGRIC

[a]Species denomination was determined in our previous study (Eremeeva *et al.*, 1993).
[b]RGRICR, Rickettsial Gamaleya Research Institute Collection, Moscow.
[c]Data for strains 3, 5, 6 are not published, but informations can be found at the RGRICR.

Table 2. Oligonucleotide primers used for genotypic identification of the Armenian isolates

Primer	Species	Gene	Nucleotide sequence (5'-3')	Amplified product size (bp)
BG 1-21			GGCAATTAATATCGCTGACGG	
	R. rickettsii	120 K antigen		650
BG 2-20			GCATCTGCACTAGCACTTTC	
Rr190.70p			ATGGCGAATATTTCTCCAAAA	
	R. rickettsii	190 K antigen		532
Rr190.602n			AGTGCAGCATTCGCTCCCCCT	
RpCS.877p			GGGGGCCTGCTCACGGCGG	
	R. prowazekii	Citrate synthase		381
RpCS.1258n			ATTGCAAAAAGTACAGTGAACA	

Cell cultures. All of the rickettsiae were cultivated in Vero cell monolayers at 32 °C in Minimal Essential Medium (Biological Industries, Kibbutz Beth Haemek, Israel) supplemented with 4% foetal bovine serum (Eurobio, Les Ulis, France).

PCR/RFLP. To prepare samples for PCR, infected and noninfected Vero cells (negative control) were washed three times in distilled water by centrifugation at 17,500 g for 5 mins and boiled for 10 mins. PCR amplification was performed using three pairs of

oligonucleotide primers: RpCS.877p and RpCS.1258n generated from the citrate synthase (CS) gene of *R. prowazekii,* Rr190.70p and Rr190.602n generated from the 190 K antigen gene of *R. rickettsii* (Regnery *et al.,* 1991), and BG1-21 and BG2-20 (BG-12) generated from the 120 K antigen gene *R. rickettsii* cell designed by B.E. Anderson (Center for Disease Control and Prevention, Atlanta). Primers were synthesized by Eurogentec (Seraing, Belgium). The nucleotide sequences of the primers are shown in Table 2. Each of the 35 cycles of amplification consisted of the denaturation at 95 °C for 20 secs, annealing at 48 °C for 30 secs, and sequence extension at 60 °C for 2 mins according to the protocol described by Regnery *et al.* (1991). 100 µl of reaction mixture, which contained 10 µl of prepared sample, 59.5 µl of distilled H_2O, 10 µl of 10 × GeneAmp^R PCR buffer II (Perkin Elmer Roche Molecular Systems, Inc., Branchburg, New Jersey, USA), 10 µl of deoxynucleotide triphosphates (2% dATP, 2% dTTP, 2% dCTP, and 2% dGTP (Eurogentec) in distilled water), 5 µl of each component of the primer pair, and 1 U/µl of AmpliTag^{R}DNA polymerase (Perkin Elmer) was prepared and processed by using a thermal cycler (PREM III, Lep Scientific, Flobio, Courbevoie, France). To verify the results of the PCR-amplifications, 5 µl of the amplified material was electrophoresed in an 1% agarose gel (Sigma) for 1 hr at 100 V. The DNA size marker set VI (*Bgl*I- and *Hinf*I-digested pBR328 DNA of Boehringer Mannheim (Meylan, France) was used.

15 – 20 µl aliquots of the amplified product were digested with 1 µl (10 – 20 U) of *Rsa*I, *Alu*I (New England Biolabs, Beverly, MA), and *Pst*I (Boehringer Mannheim) overnight at 37 °C and the restriction products were separated on 8% polyacrylamide gel at 100 V for 4 hrs, stained with ethidium bromide and detected under UV (365 nm). DNA size marker V (*Hae*III-digested pBR322 DNA) of Boehringer Mannheim was run simultaneously with the samples.

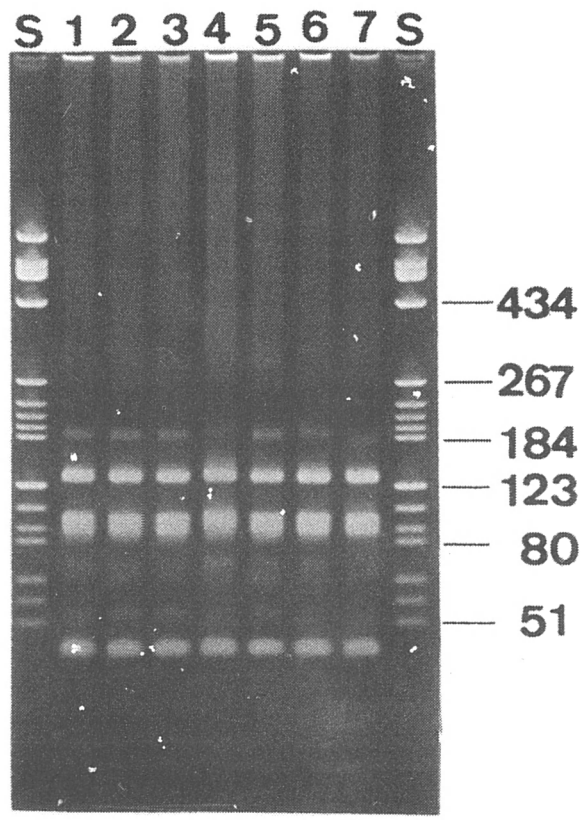

Fig. 1
PCR/RFLP analysis of rickettsial DNA by use of primer pair RpCS.877-1258pn and AluI digestion
R. slovaca strain 13-B (lane 1), Armenia 74 isolate (lane 2), Armenia 29 isolate (lane 3), *R. slovaca* strain Armenia 25 (lane 4), Armenia 5 isolate (lane 5), *R. slovaca* strain Crimea-108 (lane 6), R. sibirica strain K-1 (246) (lane 7), DNA size marker V (bp, lanes S).

Results

Atypical SFG rickettsia isolates from Armenia considered as *R. sibirica* serovariants and other atypical strains (Table 1) were genotyped by PCR/RFLP analysis with different oligonucleotide primers (Fig. 1–3).

The genotype identification with the genus-specific CS primers RpCS.877-1258pn following *Alu*I digestion allowed the separation of most SFG rickettsiae from *R. belli, R. akari, R. australis* and *R. massiliae*, and from typhus group rickettsiae. The RFLP analysis of the PCR products showed that the tested rickettsial isolates from Armenia and standard strains *R. slovaca* 13-B and *R. sibirica* K-1 had identical migration profiles consisting of 4 bands of 135, 97, 90 and 42 bp that are typical for SFG rickettsiae, including *R. rickettsii, R. conorii, R. sibirica, R. slovaca, R. rhipicephali,* and *R. montana* (Regnery *et al.,* 1991).

PCR/RFLP analysis with the species-specific Rr190.70-602pn primer pair originating from the gene of the immunodominant 190 K (omp A) antigen showed that amplicons of three isolates, Armenia 74, Armenia 29 and Armenia 5, digested with *Pst*I (Fig. 2) had identical migration profile with that of the standard strain 13-B of *R. slovaca* and recently identified *R. slovaca* strains Armenia 25 and Crimea-108. It consisted of three bands of 311, 180 and 134 bp, and differed from the standard *R. sibirica* strain K-1 (246), which consisted of three bands of 311, 134 and 90 bp. After *Rsa*I-digestion all tested and standard strains had identical profiles consisting of two bands of 240 and 119 bp, which were typical for *Rsa*I pattern polymorphism of *R. sibirica* or *R. slovaca* DNA primed with Rr190.70-602pn primer pair.

Results of the PCR/RFLP analysis of amplicons with the primer pair derived from the 120 K (omp B) antigen gene and digested with *Rsa*I (Fig. 3) showed that all tested Armenian isolates and standard strain 13-B of *R. slovaca* had identical migration profiles, namely 4 bands of 191, 163, 110 and 89 bp. The standard strain K-1 (246) of *R. sibirica* after *Rsa*I digestion revealed an other migration profile consisting of 5 bands of

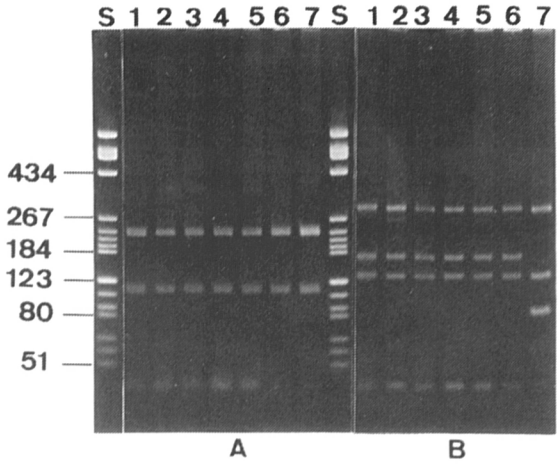

Fig. 2
PCR/RFLP analysis of rickettsial DNA by use of primer pair Rr190.70-602pn and RsaI (A) and PstI (B) digestion
For legend see Fig. 1.

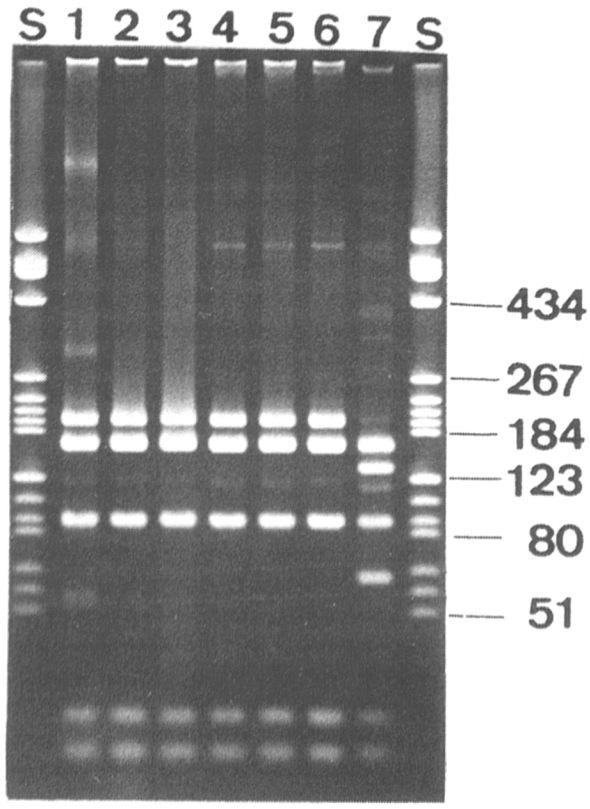

Fig. 3
PCR/RFLP analysis of rickettsial DNA by use of primer pair BG1-21 and BG2-20 and RsaI digestion
For legend see Fig. 1.

165, 132, 109, 89 and 62 bp. In our experiments the 109-110 bp *Rsa*I bands were more subtle in comparison with others, but they were detected in all obtained profiles in agreement with our previous data (Eremeeva *et al.*, 1993). Variable density of the 109-110 bp fragments in different experiments could be the results of different restriction conditions and used enzyme batch.

Discussion

In this study the SFG rickettsial strains isolated in Armenia from *D. marginatus* are identified as *R. slovaca* by PCR/RFLP analysis with primers for parts of the genes encoding the 190 and 120 K omp antigens of *R. rickettsii* used for identification of species-specificity of SFG rickettsiae isolates (Regnery *et al.*, 1991; Eremeeva *et al.*, 1993). The genotype of the identified Armenia isolates was similar to that of the reference *R. slovaca* strain 13-B (Regnery *et al.*, 1991) and previously identified *R. slovaca* strains Crimea-108 and Armenia 25 (Eremeeva *et al.*, 1993).

At present *R. slovaca* genotype identified strains are widely distributed: Slovakia (Regnery *et al.*, 1991), France (Beati *et al.*, 1992, 1993), Crimea and Armenia (Eremeeva *et al.*, 1993, and present data). In several regions *R. slovaca* occures in the same areas were other SFG rickettsiae circulate. In France and Crimea, *R. slovaca* were isolated in endemic area of Marseille spotted fever (Beati *et al.*, 1993; Eremeeva *et al.*, 1993). Moreover, rickettsiae closely related to *R. rhipicephali* (Drancourt *et al.*, 1991) and new, recently identified *R. massiliae* are prevalent in France as well (Beati and Raoult, 1993).

The characteristic peculiarity of the *R. slovaca* isolates originating from different geographical regions is their genotype homogeneity. The genotype identity of some *R. slovaca* strains (13-B, Crimea-108, Armenia 25) founded by PCR/RFLP analysis with primers for 190 K and 120 K *R. rickettsii* omp genes was confirmed by similar PFGE pattern polymorphism of the chromosomal DNA and similar protein profiles in SDS-PAGE (Eremeeva *et al.*, 1993; Roux and Raoult, 1993). The differences of *R. slovaca* strain Crimea-108 from *R. sibirica* and *R. conorii* were also revealed by Southern blot hybridization with DNA probes derived from *R. prowazekii* DNA (Balayeva *et al.*, 1993).

Until now all identified *R. slovaca* strains were isolated from *D. marginatus* considered as the primary vector and probably the reservoir of *R. slovaca.* At the same time rickettsial strains closely related to *R. slovaca* were isolated from *Ixodes ricinus* (Urvölgyi and Brezina, 1978; Řeháček *et al.*, 1972) and detected in *Haemophysalis punctata* (Řeháček *et al.*, 1972). According to these data Slovak authors suggest that *R. slovaca* can have wider spectrum of vectors. Taxonomic position of the so far unidentified isolates from

the other regions of central Europe, closely related to *R. slovaca* especially from ticks other than *D. marginatus*, need be determined.

The role of *R. slovaca* as human pathogen is unknown, except for report that *R. slovaca* could be the cause of a case of acute meningoencephalitis (Mittermayer *et al.*, 1980). At the same time, specific antibodies to SFG rickettsiae in sera of humans having been in professional contact with rural animals or their wool in Slovakia were detected (Řeháček and Tarasevich, 1988), though the other SFG rickettsioses in this country have not been diagnosed.

The antibodies to SFG rickettsiae in human sera outside the Marseille spotted fever endemic areas were detected in France (Beati *et al.*, 1993). The data on positive specific serological testing of human sera indicate the contact of the man with *R. slovaca* but probably without clinical manifestation of infection. The *R. slovaca* human pathigenicity, ecological and epidemiological significance should be further considered.

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