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GENOTYPIC AND BIOLOGICAL CHARACTERISTICS OF NON-IDENTIFIED STRAIN OF SPOTTED FEVER GROUP RICKETTSIAE ISOLATED IN CRIMEA

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Summary. – A strain of rickettsiae, designated Crimea-108, was isolated from ticks *Dermacentor marginatus* in the Crimea in 1977. Its immunobiological characteristics involve low pathogenicity for experimental animals, moderate infectivity for chick embryos, and antigenic relatedness to spotted fever group (SFG) rickettsiae (*R. sibirica, R. conorii, R. akari)*, especially to *R. sibirica.* The genotypic characterization of the strain Crimea-108 was carried out in comparison with SFG and typhus group rickettsiae by using restriction fragment length polymorphism (RFLP) analysis and DNA-probe hybridization. The marked similarity was detected between DNA restriction patterns of the strains Crimea-108, *R. sibirica* and *R. conorii*, but each of them besides comigrating fragments had specific ones. Genotypic analysis of the strain Crimea-108, the SFG and typhus group rickettsiae by three independent DNA probes, based on *R. prowazekii* DNA, gave unique hybridization patterns for the Crimea-108 strain with all probes. The obtained data show that the Crimea-108 isolate does not belong to the species of *R. sibirica, R. conorii, R. akari.* The strain Crimea-108 is a novel strain of SFG rickettsiae for the Crimea region.

Key words: Crimea isolate; spotted fever group rickettsiae; genotypic characteristic

Introduction

Development of new methods for typing of typhus group and SFG rickettsiae is based on the analysis of RFLP of the genome DNA along with DNA probe (Regnery, 1991; Demkin *et al.*, 1991) as well as on the RFLP analysis of DNA products amplified in the polymerase chain reaction (PCR) using oligonucleotide primers of various specificity (Regnery, 1990; Regnery *et al.*, 1991). The obtained data of genotyping of prototype rickettsial strains and species are in a correlation with the modern classification of species (Weiss and Moulder,

1984), expanding it with genotypic characteristic of strain within a rickettsial species (Regnery *et al.*, 1986; Regnery, 1990; Artemiev *et al.*, 1991). The correlation between the findings of the genotypic identification and available classification of the genus Rickettsia allows to apply the genotypic characteristic to identification of rickettsial isolates non-identified completely by currently used methods. The latter is specially important for SFG rickettsiae due to manifest antigenic similarity of these agents which complicates their identification by serologic methods.

The greater interest shown at present to study SFG rickettsiae can be explained by an increased incidence of rickettsial diseases in humans (Wisseman, 1983; Mansueto *et al.*, 1986), a detection of new types of foci, and in the areas that were not endemic before (Uchida *et al.*, 1985; Shaked *et al.*, 1988; Balayeva and Ignatovich, 1989; Makarova and Tarasevich, 1989), an isolation of large quantity of strains among which new species of SFG rickettsiae have been identified (Weiss and Moulder, 1984). Some of the isolates need further identification. Among them there is a group of low pathogenic strains which are antigenically related to SFG rickettsiae and were isolated both Europe and Asia, out of the limits of nosoarea of tick-borne typhus of northern Asia in the areas of *R. sibirica* ticks-vector distribution (Brezina *et al.*, 1969; Yablonskaya, 1978; Makarova *et al.*, 1978; Vorontsova *et al.*, 1980; Řeháček and Tarasevich, 1988). The isolated strains are distinguishable in their antigenic relation to *R. sibirica* and *R. conorii.* On the basis of their serologic specificity some of strains isolated in Slovakia were considered to be a separate species *R. slovaca* (Urvölgyi and Brezina, 1978) that has been supported by the results of genotypic identification (Regnery *et al.*, 1991). The exact species identification of other strains isolated in this areas has not yet been established. In the Crimea a rickettsial strain designated Crimea-108 was isolated from ticks *Dermacentor marginatus,* and it was preliminarily characterized as a low pathogenic strain antigenically related to *R. sibirica* (Vorontsova *et al.*, 1980).

In the present paper we describe some genotypic and biological characteristics of the Crimea-108 isolate and show its genotypic differences from *R. sibirica, R. conorii* and *R. akari.*

Materials and Methods

Rickettsial strains. The strain Crimea-108 was isolated by T. A. Vorontsova (N. F. Gamaleya Research Institute, Moscow) in 1977 from ticks *Dermacentor marginatus* in the Crimea. A culture of the strain Crimea-108 in yolk sacs of chick embryos (CE) was obtained by their inoculation with suspension of the second laboratory generation of ticks. The strain Crimea-108 (8th-11th passage in CE) was studied in comparison with various rickettsial strains (species) (Table 1).

All the rickettsial strains were cultivated in yolk sacs of CE and purified as described by Aniskovich *et al.* (1989). Rickettsiae of typhus group were inactivated with 0.1 % formalin at 6 °C for 24 hrs before purification. Rickettsiae of SFG were purified without inactivation.

The pathogenicity of the strain Crimea-108 was examined in experimental outbred animals: male guinea pigs, white rats, white mice. Antigenic properties of the strains were studied by using soluble

Table 1. Rickettsial strains employed in the study

Species	Strains	Source	Origin	Year of isolation
	Crimea-108	*D. marginatus*	Crimea	1977
R. sibirica	Netsvetaev (232)	Patient	West Siberia	1946
R. sibirica	K-1 (246)	*D. nuttalii*	East Siberia	1949
R. sibirica	Altay-81/88	*D. silvarum*	West Siberia	1988
R. sibirica	Gornyi-54/58	*D. nuttalii*	West Siberia	1988
R. conorii	ITT (Indian tick typhus)	*R. sanguineus*	Kashmir, India	1950
R. akari	MK (Kaplan)	Patient	New York, USA	1950
R. akari	M-3	*Mus musculus*	Ukraine	1950
R. canada	2678	*H. leporis-palustris*	Canada	1963
R. typhi	Ger	Patient	Batumi, Georgia	1946
R. prowazekii	Breinl	Patient	Warsaw, Poland	1919–1921

antigens of rickettsiae, corpuscular antigen of *C. burnetii* and sera of infected animals by using the standard micromethod of the complement fixation reaction (micro-CFR).

Total rickettsial DNA was isolated by the method of Priefer *et al.* (1984).

Restriction endonuclease digestion and electrophoresis. Rickettsial DNA was digested with restriction endonucleases *Hind*III, *Eco*RI, *Msp*I and *Pst*I as recommended by Maniatis *et al.* (1982). For the purpose of RFLP analysis the DNA digests were electrophoresed on 0.6–0.7 % agarose gels (agarose type II, Sigma) with TAE buffer at 4 V/cm for 2–3 hrs at the beginning, then for 1–2 days at 1 V/cm. The amounts of DNA loaded on each lane of the gel varied from 1.5 to 2 mkg. For the purpose of blot-hybridization the DNA digests were electrophoresed on 0.8 % agarose gels with TBE buffer at 3.5 V/cm for 3 hrs. Phage lambda DNA, cleaved with *Hind*III restriction endonuclease was used for DNA fragment size standards. After electrophoresis the gels were stained with ethidium bromide and photographed in UV light.

Probe preparation and hybridization. PBH11 and PBH13 probes were morphospecific *Hind*III-derived DNA fragments from *R. prowazekii* (Demkin *et al.*, 1991). MW264 probe was *Eco*RV-derived DNA fragment containing *R. prowazekii* citrate-synthase gene (Wood *et al.*, 1987). 50–100 ng of each probe was labelled with ^{35}S-deoxycitidine (Amersham), using an oligolabelling kit (Pharmacia) according to the manufacturer's instruction. The DNA digests from agarose gels were blotted overnight to Zeta-Probe membranes (Bio-Rad) by an alkaline procedure. DNA-probes were hybridized with blots in 30 % formamide (Serva) buffer at 42 °C. Blotting, hybridization and washing of the membranes were carried out according to the manufacturer's (Bio-Rad) instructions.

Results and Discussion

The biological properties of the strain Crimea-108 were characterized by peculiarities of its growth in CE and its virulence for intraperitoneally (ip) infected male quinea pigs, white rats and white mice.

Rickettsiae of the strain Crimea-108 grew in 5 days-old CE with moderate multiplication in yolk sac tissue causing the death of CE 4–6 days after inoculation. Unlike rickettsiae of SFG, the soluble antigen obtained from CE

cultures of the strain Crimea-108 had weak antigenic activity in CFR with homologous serum (up to 1:5).

The reaction of guinea pigs to the ip inoculation of the strain Crimea-108 depended on the size of the infectious dose. Guinea pigs inoculated with 10^5–10^3 ID_{50} showed short fever (2–3 days) and mild scrotal swelling in some animals. Lower infectious doses (10^2–10^0 ID_{50}) induced an inapparent infection with seroconversion. High titers of CF antibodies with homologous antigen (to 1:160) were determined in animals infected with 10^5 ID_{50}. In other infected guinea pigs (10^4–10^0 ID_{50}) the titers of CF antibodies were not higher than 1:20.

White rats and white mice responded to the inoculation with 10^5–10^4 ID_{50} of the strain Crimea–108 only serologically without any signs of infection. Titers of CF antibodies with homologous antigen were within the range of 1:5 - 1:20.

The antisera of experimental animals infected with the strain Crimea-108 showed cross-reactivity with heterologous antigens of *R. sibirica, R. conorii* and *R. akari* (Table 2). The guinea pig antisera to the strain Crimea-108 reacted at similar titers (1:160) with homologous antigen and the antigens of *R. sibirica, R. conorii* and *R. akari.* The sera of white rats reacted in CFR with homologous antigen and the antigen of *R. sibirica* at similar titers, and at a lower titer with the antigen of *R. conorii*, and they did not react with the antigen of *R. akari.* In the mouse sera there were CF antibodies in low titers (1:5 - 1:10) to its homologous antigen and that of *R. sibirica* only.

For further characterization of the antigenic relationships of the strain Crimea-108 to SFG rickettsiae we performed cross-tests of mouse antisera to prototype strains of SFG rickettsiae with soluble antigen of Crimea-108 rickett-

Table 2. Results of serotyping of the strain Crimea-108 obtained by use of antigens and antisera of infected guinea pigs, white rats and white mice in micro-CFR

Soluble rickettsial antigens	Antibody titers with antigens					
	Crimea-108			*R. siberica* (Netsvetaev)	*R. conorii* (ITT)	R. akari (MK)
	guinea pigs	white rats	white mice	white mice		
Strain Crimea-108	160*	20	5–10	5	<5**	<5
R. sibirica (Netsvetaev)	160	20	5–10	40	10	<5
R. conorii (ITT)	160	5	<5	10	40	<5
R. akari (MK)	160	<5	<5	<5	<5	40

*Reciprocals of serum titers
**Negative reaction in the dilution of serum 1:5

siae (Table 2). The mouse antisera to *R. conorii* and *R. akari* did not show cross-reactivity to the Crimea-108 antigen. The mouse antisera to *R. sibirica* reacted with the Crimea-108 antigen at a lower titer than with the homologous one.

The sera of all experimental animals did not react with antigens of typhus group rickettsiae and of *C. burnetii.*

The obtained immuno-biologic characteristics of rickettsiae of the strain Crimea-108 involve low pathogenicity for experimental animals (guinea pigs, white rats and white mice) and moderate infectivity for CE. The serological analysis by CFR showed that this strain is antigenically related to SFG rickettsiae (*R. sibirica, R. conorii, R. akari*), especially to *R. sibirica.*

The genotypic characterization of the strain Crimea-108 was carried out in comparison with SFG rickettsiae – *R. sibirica, R. conorii, R. akari* and typhus group rickettsiae – *R. prowazekii, R. typhi, R. canada* by use of RFLP analysis and DNA-probe hybridization.

The strain Crimea-108 was analyzed by RFLP method in comparison with *R. sibirica, R. conorii* and *R. prowazekii* using endonucleases *Hind*III, *Msp*I and *Pst*I (Fig. 1). This analysis revealed various distinctions in the DNA restriction patterns of the compared agents, which were clearly seen in the high molecular weight zone. Considerable differences in the DNA restrictions patterns of the strains Crimea-108 and *R. prowazekii* were found. A marked similarity was detected between DNA restriction patterns of the strains Crimea-108, *R. sibirica* and *R. conorii*, but each of them besides comigrating fragments had specific fragments. The observed differences between the strains Crimea-108 and *R. sibirica* in RFLP should be regarded as significant. Since the great number of *R. sibirica* strains (13 strains including the strains studied in this experiment) was identical when analyzed with the same restriction endonucleases (Zhao and Fan, 1990; Balayeva *et al.*, in press). As to the differences between the strains Crimea-108 and *R. conorii* they are not demonstrative, because *R. conorii* strains themselves are known to have definite genotypic differences (Regnery, 1991).

The differences between the genotypes of the strain Crimea-108, the SFG rickettsiae (*R. sibirica, R. conorii, R. akari*) and the typhus group rickettsiae (*R. prowazekii, R. typhi, R. canada)* were detected by using DNA probe hybridization to *Hind*III or *Eco*RI digested rickettsial DNA. PBH11, PBH13 and MW264 probes, derived from *R. prowazekii* DNA were used. Each of these probes formed a specific hybridization pattern with blotted DNA that allowed to differentiate the rickettsiae of typhus group to the species level, to distinguish the members of typhus group rickettsiae from SFG rickettsiae as well as *R. akari* from *R. sibirica* and *R. conorii* (Demkin *et al.*, 1991). The probe PBH11 was used to hybridize the *Hind*III and *Eco*RI DNA digests of the compared strains of rickettsiae (Fig. 2). It was found that the hybridization zones of *Hind*III DNA digests of the strains Crimea-108 and *R. prowazekii* were localized closely to each other and differed from those of *R. conorii, R. akari, R. sibirica, R. canada* and *R. typhi.* When the DNA probe PBH11 was used to test *Eco*RI DNA digests the hybridization zones of the strains Crimea-108 and *R. prowazekii* had different

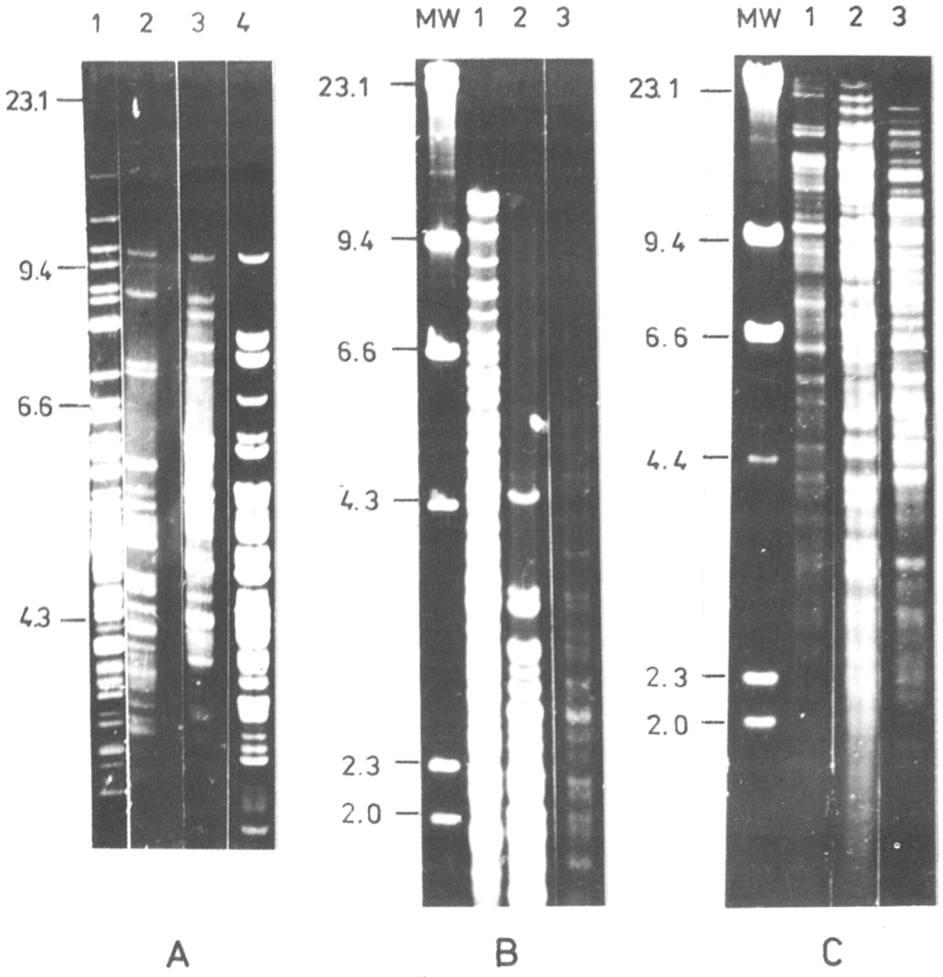

Fig. 1

Restriction endonuclease digestion of DNA of different rickettsial strains

A: *Hind*III-digested DNA of *R. prowazekii* strain Breinl (lane 1), Crimea-108 (lane 2), *R. conorii* strain ITT (lane 3), and *R. sibirica* strain K-1 (lane 4).

B: *Msp*I-digested DNA of *R. prowazekii* strain Breinl (lane 1), *R. sibirica* strain K-1 (lane 2), and Crimea-108 (lane 3). *Hind*III-digested lambda DNA (lane MW).

C: *Pst*I-digested DNA of *R. sibirica* strain Gornyi (lane 1), Crimea-108 (lane 2), and *R. prowazekii* strain Breinl (lane 3). *Hind*III-digested lambda DNA (lane MW).

Kbp values of molecular weight standards are shown on the left side of the gels.

localization; the other strains also formed different patterns.

Differences between the compared rickettsiae were also detected by the probe PBH13 with *Hind*III digests (data not shown).

The probe MW264 that contains the *R. prowazekii* citrate-synthase gene (Wood *et al.*, 1987) was used to test *Hind*III DNA digests (Fig. 3). This DNA probe also formed hybridization zones that differed in localization for the strain Crimea-108 and strains of *R. sibirica, R. conorii, R. akari, R. prowazekii, R. typhi, R. canada.*

Thus the obtained data show that the strain Crimea-108 is related antigenically to the SFG rickettsiae, especially to *R. sibirica,* and it produces DNA restriction patterns similar to *R. sibirica* and *R. conorii.* However, the genotypic analysis by DNA probe hybridization with a set of the independent *R. prowazekii* derived DNA probes revealed genotypic differences between the isolate Crimea-108 and *R. sibirica, R. conorii* and *R. akari* references strains. Our findings support the

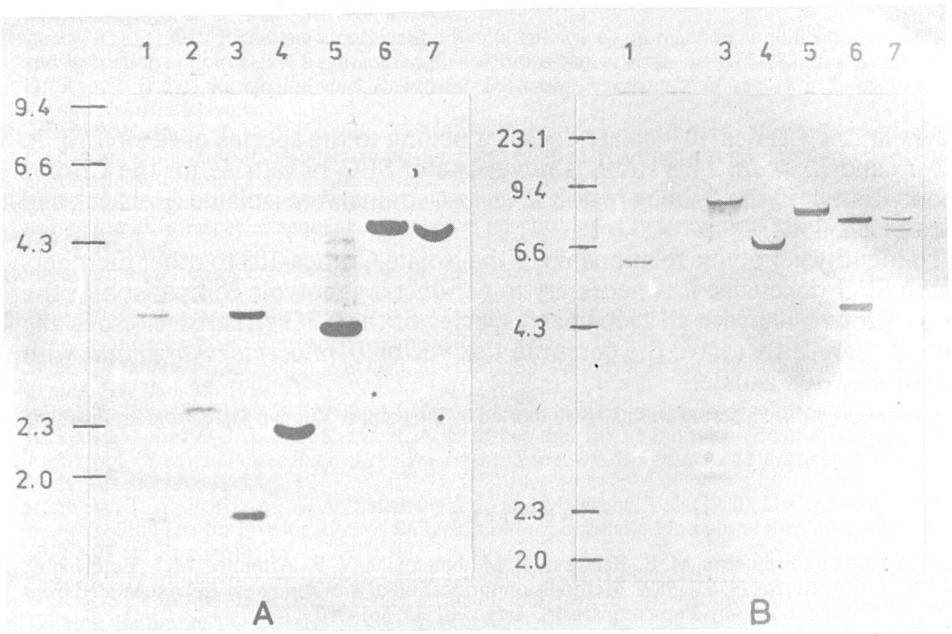

Fig. 2

Southern blot hybridization analysis of DNA of different rickettsial strains with PBH11 probe A: *Hind*III-digested DNA of *R. conorii* strain ITT (lane 1), *R. akari* strain M-3 (lane 2), *R. sibirica* strain K-1 (lane 3), *R. canada* strain 2678 (lane 4), *R. typhi* strain Ger (lane 5), *R. prowazekii* strain Breinl (lane 6), and Crimea-108 (lane 7). B: *Eco*RI-digested DNA of *R. conorii* strain ITT (lane 1), *R. sibirica* strain K-1 (lane 3), *R. canada* strain 2678 (lane 4), *R. typhi* strain Ger (lane 5), *R. prowazekii* strain Breinl (lane 6), and Crimea-108 (lane 7).

Kbp values of molecular weight standards are shown on the left side of blots.

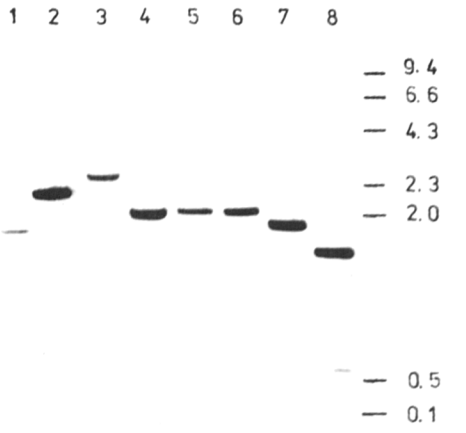

Fig. 3
Southern blot hybridization analysis of DNA of different rickettsial strains with MW264 probe
*Hind*III-digested DNA of *R. akari* strain M-3 (lane 1), *R. prowazekii* strain Breinl (lane 2), Crimea-108 (lane 3), *R. conorii* strain ITT (lane 4), *R. sibirica* strain Altay 81/88 (lane 5), *R. sibirica* strain K-1 (lane 6), *R. canada* strain 2678 (lane 7), and *R. typhi* strain Ger (lane 8).
Kbp values of molecular weight standards are shown on the right side of the blot.

view that the Crimea-108 isolate does not belong to the species of *R. sibirica, R. conorii* and *R. akari.* This strain is novel one of SFG rickettsiae for the Crimea region. Earlier in the Crimea region *R. conorii* strains were isolated (Řeháček and Tarasevich, 1988).

To identity genotypic relationship of the isolate Crimea-108 to other members of the SFG rickettsiae it is necessary to conduct a genotypic comparison of the strain Crimea-108 with all recognized species of the SFG rickettsiae, especially with *R. slovaca* that have the common tick vector *Dermacentor marginatus* with the Crimea-108 isolate.

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