# GENOTYPIC CHARACTERIZATION OF RICKETTSIAE BY DNA PROBES GENERATED FROM *RICKETTSIA PROWAZEKII* DNA

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**Summary.** – Southern blot analysis of *Hind*III-cleaved rickettsial DNA was used for genotypic characterization of the typhus group (TG) species (*R. prowazekii, R. typhi, R. canada*) and a few species of the spotted fever group (SFG) rickettsiae (*R. sibirica, R. conorii, R. akari*). Four different DNA probes were employed. PBH11 and PBH13 probes were morphospecific *Hind*III fragments of *R. prowazekii* DNA. MW218 probe contained the gene for 51 K antigen and MW264 probe contained the citrate synthase gene of *R. prowazekii.* All the probes hybridized with the tested TG and SFG rickettsial DNAs, forming from 1 to 5 bands, but they did not with *R. tsutsugamushi* or *C. burnetii* DNAs. All the probes demonstrated specific hybridization patterns with TG species and *R. akari*. PBH11, PBH13 and MW264 probes clearly distinguished *R. sibirica* and *R. conorii* from the other tested rickettsiae, but not from each other. However, these two species differed slightly with MW218 probe. Several strains of each species were analyzed in this way and except for strains of *R. conorii* identical intraspecies patterns were obtained. These data lead us to consider the obtained hybridization patterns as criteria for genotypic identification.

**Key words:** rickettsiae; genotyping; Southern blot; DNA probes

## Introduction

Methods of molecular genetics have been recently applied to the study of the genetic relationship between rickettsiae, introducing genotyping as a tool of identification and differentiation of rickettsiae. In general techniques of study of genetic structure of rickettsiae are based on electrophoretic analysis of fragments, generated by restriction endonuclease cleavage of DNA. This approach, now often referred to as restriction fragment length polymorphism (RFLP) analysis is applied to whole genome or its specific segments amplified by the polymerase chain reaction (PCR/RFLP). Specific DNA sequences can be detected also by specific DNA probes using the Southern blot hybridization analysis (Regnery 1990, 1991). By this manner distinct differences in the structure of chromosomal DNA have been observed not only between the established rickettsial species, but also between some isolates within several species considered earlier as homogenous. Despite much progress achieved in this way our knowledge of genetic diversity of rickettsial species is yet poor at present and the currently available genotypic data are not sufficient to introduce them as taxonomic criteria. The central problem which remains unsolved is the choice of a segment of rickettsial genome with appropriate differential characteristics and its certification for specificity with regard to different species and different isolates of the same species.

Earlier we have used RFLP analysis to study structural variability of chromosomal DNA of TG rickettsiae (Balayeva *et al.*, 1989; Rydkina *et al.*, 1990; Artemiev *et al.*, 1991). On the basis of observed distinctions in electrophoretic pattern many incomigrating fragments of different TG rickettsiae have been identified, which were referred to as "morphospecific". Despite the unknown genetic structure of these fragments they may be used as DNA probes.

In this paper we report the results of typing of TG and a few species of SFG rickettsiae by four different DNA probes. Two of them contained "morphospecific" fragments of *R. prowazekii* DNA and two others contained recognized genes of *R. prowazekii,* relatively conservative citrate synthase gene and relatively variable gene for 51 K antigen (Krause *et al.*, 1985; Wood *et al.*, 1987). Preliminary results of this study were already published (Demkin *et al.*, 1991).

## Materials and Methods

*Rickettsial strains* and their characteristics are presented in Table 1. Rickettsiae were cultivated in chick embryos, infected yolk sacs were collected and stored at –60 °C.

**Table 1 Rickettsial species and strains**

| Species/Strain | Original source | Year of isolation |
|---|---|---|
| *R. prowazekii* | | |
| Breinl, ATCC VR-142 | patient, Warsaw, Poland | 1922 |
| Chernikova | patient, Minsk, Byelorussia | 1967 |
| E, ATCC VR-233 | spontaneous low virulent mutant of strain Madrid I | 1943 |
| EVir | virulent mutant of strain E | 1969 |
| Kuzina | patient, Moscow, Russia | 1950 |
| L | patient, Lvov, Ukraine | 1946 |
| Vladyko | patient, Minsk, Byelorussia | 1968 |
| *R. typhi* | | |
| B-1 | *Rattus norvegicus*, Batumi, Georgia | 1946 |
| Ger | patient, Batumi, Georgia | 1946 |
| Museibov | patient, Baku, Azerbaijan | 1949 |
| Wilmington, ATCC VR-144 | patient, North Carolina, USA | 1928 |
| X | *Xenopsylla cheopis*, Batumi, Georgia | 1946 |
| *R. canada* | | |
| 2678, ATCC VR-610 | *Haemaphysalis leporispalustris* Ontario, Canada | 1963 |
| *R. sibirica* | | |
| Altay-81/88 | *Dermacentor silvarum*, West Siberia, Russia | 1988 |
| Chyta-18/85 | *Dermacentor nuttali*, East Siberia, Russia | 1985 |
| Gornyi-54/88 | *D. nuttali*, East Siberia, Russia | 1988 |
| K-1 (246), ATCC VR-151 | *D. nuttali*, East Siberia, Russia | 1949 |
| Netsvetaev (232) | patient, West Siberia, Russia | 1946 |
| Primorie-20/84 | *Haemaphysalis concinna*, Far East, Russia | 1984 |
| *R. conorii* | | |
| Itt, ATCC VR-597 | *Rhipicephalus sanguineus*, Kashmir, India | 1950 |
| Ktt | *Haemaphysalis leachi*, Nairobi, Kenya | 1953 |
| M-1 | *R. sanguineus*, Sukhumi, Abkhazia | 1947 |
| *R. akari* | | |
| M-3 | *Mus musculus*, Donetsk, Ukraine | 1950 |
| MK(Kaplan), ATCC VR-148 | patient, NY, USA | 1946 |
| *R. tsutsugamushi* | | |
| Gilliam, ATCC VR-312 | patient, Assam-Burma, Indo-China | 1944 |
| *C. burnetii* | | |
| M-44 | low virulent strain, selected from Italo-Greek strain Grita | 1954 |

*Purification of rickettsiae.* All steps of the purification procedures were carried out at 4 °C. These buffers were used: HE buffer – 10 mmol/l HEPES (Serva), 5 mmol/l EDTA (Serva), pH 7.4, SHE buffer – 7.5 % sucrose (Serva) in HE buffer, TE buffer – 10 mmol/l Tris HCl, 1 mmol/l EDTA, pH 8.0.

The TG rickettsiae were inactivated with formalin and purified as described earlier (Aniskovich *et al.*, 1989). The SFG rickettsiae were purified without inactivation. The procedure adopted from Aniskovich *et al.* (1989) consisted from disintegration of yolk sacs by shaking with glass beads, two cycles of centrifugation through 25 % sucrose, magnesium treatment and filtration through AP20 filter (Millipore).

The purification of *C. burnetii* and *R. tsutsugamushi* was carried out as follows. Formalin inactivated yolk sacs were disintegrated by shaking with glass beads, mixed with 3 volumes of 0.9 % NaCl in HE buffer and centrifuged at 680 × g for 15 mins. The supernatant was loaded over an equal volume of sucrose solution (35 % sucrose in HE buffer for *C. burnetii*, 15 % sucrose in HE buffer for *R. tsutsugamushi*) and centrifuged at 24,000 × g for 40 mins. In the case of *C. burnetii* the pellet was resuspended in 0.9 % NaCl, centrifuged at 10,400 × g for 15 mins and the last pellet was resuspended in TE buffer. In the case of *R. tsutsugamushi* the pellet after the sucrose cushion centrifugation was resuspended in SHE buffer, loaded over 3 volumes of 40 % Percoll (Pharmacia-LKB) in 0.25 mmol/l sucrose, and centrifuged at 42,000 × g for 40 mins in Beckman 55.2 Ti rotor. The rickettsiae containing band was collected and diluted with SHE buffer. The rickettsiae were washed with SHE buffer by 2-3 cycles of centrifugation at 8 000 × g for 20 mins to eliminate Percoll. The final pellet was resuspended in SHE buffer.

*Preparation of rickettsial DNA* was described previously (Balayeva *et al.*, 1989).

*Restriction analysis and blot hybridization.* Restriction endonucleases were purchased from Ferment (Vilnius, Lithuania).Electrophoresis was carried out in 0.8 % agarose slab gel (Ultra Pure, DNA grade, BioRad) containing ethidium bromide in 1 × TBE buffer. 0.4 – 0.7 μg of DNA was loaded per track. After electrophoresis the gel was photographed in UV light and DNA fragments were transferred to Zeta-Probe membrane (BioRad). Blotting, hybridization and washing of the membranes were carried out according to the manufacturer's (BioRad) instructions.

*Probes* PBH11 and PBH13 were morphospecific *Hind*III fragments of *R. prowazekii* DNA of the size of about 3.8 and 4.8 kbp, respectively (Balayeva *et al.*, 1993). Probe MW264 was an *Eco*RV fragment (2 kbp) harboring the citrate synthase gene of *R. prowazekii* (Wood *et al.*, 1987). Probe MW218 was a *Sal*I fragment (7 kbp) containing the 51 K protein gene of *R. prowazekii* and it was derived from recombinant plasmid pMW218d (Krause *et al.*, 1985). DNA fragments being used as probes were isolated by preparative electrophoresis, recovered from agarose gel by electroelution, ethanol precipitated and redissolved in TE buffer. DNA probes were labelled with $^{32}P$ or $^{35}S$ using an oligolabelling kit (Pharmacia) according to manufacturer's instructions.

discriminated. E.g. *R. prowazekii* and *R. typhi* displayed very close bands (approximately at 4.0 and 3.7 kbp. The same problem appeared with *R. sibirica* and *R. akari* (Table 2).

The MW264 probed DNAs of nearly all the tested rickettsial species were characterized by one band in the area of 2 – 2.5 kbp (Table 2). This fact appears to reflect a high conservatism of the citrate synthase gene region of rickettsial genome (Regnery *et al.*, 1991), but it makes the probe inappropriate for the purpose of differentiation of rickettsiae.

Probe MW218 formed most complicated patterns of hybridization (Fig. 1B). The number of bands varied from

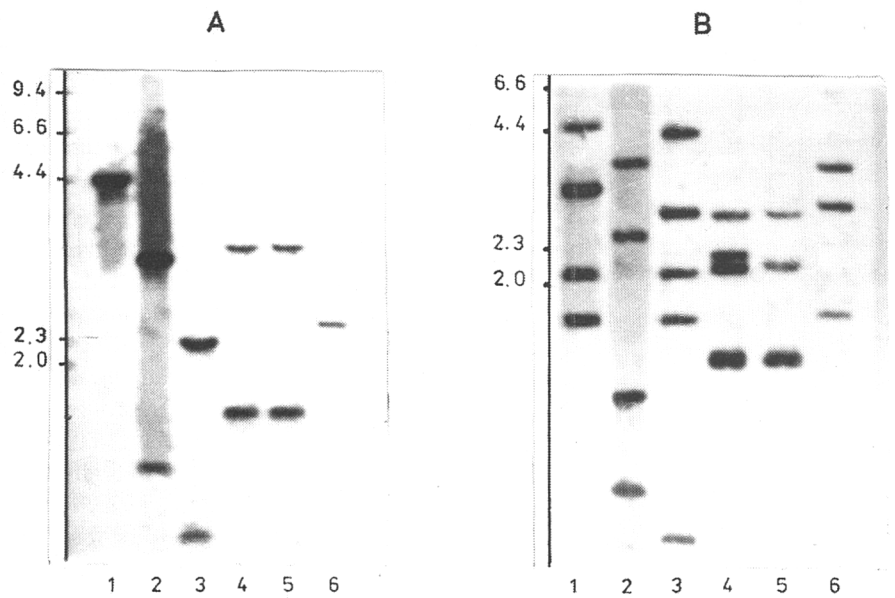


**Fig. 1**
**Southern blot hybridization analysis of HindIII-cleaved DNA of standard rickettsial strains with probes PBH11 (A) and MW218 (B)**
*R. prowazekii* strain Breinl (lane 1), *R. typhi* strain Wilmington (lane 2), *R. canada* strain 2678 (lane 3), *R. sibirica* strain K-1 (lane 4), *R. conorii* strain M-1 (lane 5), *R. akari* strain M-3 (lane 6). kbp values of *Hind*III fragments of lambda DNA used as standards are shown on the left side of the blots.

## Results

At the first stage we typed standard strains of each species: *R. prowazekii* - Breinl, *R. typhi* - Wilmington, *R. canada* - 2678, *R. sibirica* - K-1, *R. conorii* - M-1, *R. akari* - M-3. All the used DNA probes (PBH11, PBH13, MW218, MW264) gave rise to hybridization signals with tested DNA of TG and SFG rickettsiae, forming from 1 to 5 bands on autoradiographs (Fig. 1, Table 2).

Probe PBH11 was most resolvable for TG species and *R. akari*. *R. sibirica* and *R. conorii* were clearly distinguished from other tested rickettsiae but not from each other by this probe (Fig. 1A). Probe PBH13 behaved in a similar manner. However, some species produced very similar patterns that could be hardly

3 to 5 for different species, that may be obviously attributed to large size of the probe. Whereas some hybridization bands were coincident, others were non-coincident. E.g. *R. prowazekii* and *R. canada* had two coincident bands of approximately 2.1 and 1.7 kbp. There were coincident bands even for some species of TG and SFG rickettsiae (2.6 – 2.7 kbp for *R. canada, R. sibirica, R. conorii* and *R. akari,* and 2.1 – 2.2 kbp for *R. prowazekii, R. canada, R. sibirica* and *R. conorii*), though the total bands patterns were species specific. These data indicate that there are both high conservative and variable sequences in rickettsial genomes, which are homologous to probe MW218. It should be pointed out that by means of probe MW218 all tested rickettsial species could be discriminated.

**Table 2. Hybridization patterns of HindIII-cleaved rickettsial DNAs**

| Probes | Rickettsial species | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *R. prowazekii* | | *R. typhi* | | *R. canada* | | *R. sibirica* | | *R. conorii* | | *R. akari* | |
| | No. | kbp | No. | kbp | No. | kbp | No. | kbp | No. | kbp | No. | kbp |
| PBH11 | 1 | 4.0 | 2 | 3.1<br>1.5 | 2 | 2.3<br>1.2 | 2 | 3.4<br>1.8 | 2 | 3.4<br>1.8 | 1 | 2.5 |
| PBH13 | 1 | 4.0 | 1 | 3.7 | 2 | 1.7<br>0.6 | 1 | 4.8 | n.d. | | 1 | 5.2 |
| MW264 | 1 | 2.4 | 2 | 1.8<br>0.7 | 1 | 2.0 | 1 | 2.2 | 1 | 2.2 | 1 | 2.0 |
| MW218 | 4 | 4.4<br>2.9<br>2.1<br>1.7 | 4 | 3.5<br>2.5<br>1.2<br>0.8 | 5 | 4.2<br>2.6<br>2.1<br>1.7<br>0.7 | 5 | 2.6<br>2.3<br>2.2<br>1.5<br>1.4 | 4 | 2.6<br>2.2<br>1.5<br>1.4 | 3 | 3.3<br>2.7<br>1.8 |

No. – number of hybridization bands; kbp – size of hybridization bands in kbp; n.d. – not done

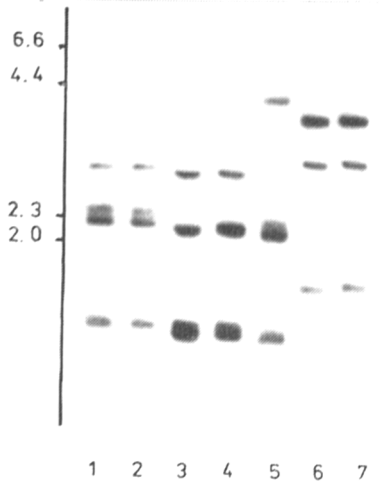


**Fig.2**
**Southern blot hybridization analysis of HindIII-cleaved DNA of different strains of SFG rickettsiae with probe MW218**
*R. sibirica* strain Altay (lane 1), *R. sibirica* strain K-1 (lane 2), *R. conorii* strain M-1 (lane 3), *R. conorii* strain Ktt (lane 4), *R. conorii* strain Itt (lane 5), *R. akari* strain M-3 (lane 6), *R. akari* strain MK (lane 7). kbp values of *Hind*III fragments of lambda DNA used as standards are shown on the left side of the blot.

Probes PBH11, PBH13 and MW264 distinguished *R. sibirica* and *R. conorii* from other tested rickettsiae, but they did not distinguish these two species from each other (Fig. 1, Table 2).

To determine whether the hybridization patterns obtained with standard rickettsial strains are peculiar also to other representatives of the same species, a set of experiments was conducted, in which several strains of the same species were tested by probes PBH11 and MW218. By means of probe PBH11 there were typed 7 strains of *R. prowazekii* (Breinl, E, EVir, Kuzina, L, Vladyko, Chernikova), 5 strains of *R. typhi* (B-1, Ger, Museibov, X, Wilmington) and 5 strains of *R. sibirica* (Altay, Gornyi, K-1, Primorie, Chyta). In all cases the hybridization patterns were identical for different strains of the same species. By probe MW218 there were tested 6 strains of *R. prowazekii* (Breinl, E, EVir, Katsynian, Kuzina, Chernikova), 3 strains of *R. typhi* (Ger, Museibov, Wilmington), 2 strains of *R. sibirica* (Altay, K-1), 3 strains of *R. conorii* (M-1, Itt, Ktt) and 2 strains of *R. akari* (M-3, MK). In the case of TG species all tested strains had identical hybridization patterns (data not shown), but with SFG rickettsiae this was true only for *R. sibirica* and *R. akari,* but not for *R. conorii (Fig. 2). R. conorii* Itt was distinguishable from strains Ktt and M-1 by one of the four hybridization bands.

General specificity of the used probes was examined with DNAs from various sources. Probes PBH11, PBH13, MW218 and MW264 did not react with DNAs prepared from uninfected yolk sacs, *E. coli* and phage lambda (data not shown). This indicates that the observed signals were specific for rickettsial DNA. All these probes did not reveal any homology to *C. burnetii* DNA. Probes PBH11, MW264 and MW218 did not hybridize to *R. tsutsugamushi* DNA as well (probe PBH13 was not tested). Probe PBH11 did not react with DNAs of *Bordetella pertussis, B. parapertussis, Brucella melitensis, Legionella pneumophilla* (2 strains), *L. gordanis, Vibrio cholerae, Yersinia enterocolitica, Y. pseudotuberculosis, Y. pestis, Shigella zonnei* and *Francisella tularensis* (2 strains).

## Discussion

Southern blot analysis of total DNA or specific DNA segments amplified by PCR has been employed for genotypic characterization of various rickettsial species and

isolates predominantly from SFG (Regnery *et al.*, 1985; Regnery *et al.*, 1986; Oaks *et al.*, 1989; Tringali *et al.*, 1989; Fuerst *et al.*, 1990; Ralph *et al.*, 1990; Spruill, Regnery 1990; Drancourt *et al.*, 1991; Gilmore, Hackstadt 1991; Regnery 1991; Regnery *et al.*, 1991; Manor *et al.*, 1992). Efficiency of these techniques depends on the degree to which the selected genomic areas, detected by probes or primers, reflect real differences between the species. Our knowledge of the structure of rickettsial chromosomal DNA is very imperfect to decide, which of the revealed genetic differences should be used as markers of intraspecies or interspecies deviations, the more so as only a limited number of strains of the same species has been genotyped in most cases. In this regard the search for a segment of rickettsial genome with appropriate differential characteristics still continues.

The most common view of genetic structure and genetic relatedness of rickettsiae can be taken from RFLP analysis of the whole chromosomal DNA on the basis of simple or pulse electrophoresis. Such analysis has been used to discriminate both species and intraspecies isolates of rickettsiae (Artemiev *et al.*, 1991; Regnery, Spruill 1984; Regnery *et al.*, 1983; Balayeva *et al.*, 1989; Rydkina *et al.*, 1990). A number of DNA fragments has been revealed, which were unique for a given species or isolate ("morphospecific" fragments). The use of morphospecific fragments as probes in Southern blot analysis gave different patterns at least for those samples, which were different in morphospecific fragment destination (Regnery *et al.*, 1986).

As probes PBH11 and PBH13 we employed two *Hind*III fragments of *R. prowazekii* DNA, which had no opposites in *Hind*III digests of *R. typhi* and *R. canada.* Two other probes (MW218 and MW264) were chosen on the basis of phenotypic features (Krause *et al.*, 1985; Wood *et al.*, 1987). All these probes were used for genotypic characterization of TG rickettsiae from each other and from SFG rickettsiae. They hybridized to TG and SFG representatives of genus Rickettsia but they did not to *R. tsutsugamushi.* The absence of hybridization signals with *R. tsutsugamushi* DNA is not surprising. The taxonomic status of this agent has to be revised because its biological and biochemical characteristics are different from those of the other members of the genus (Weiss and Moulder, 1984; Tamura, 1991).

For each assayed TG species all the probes formed specific hybridization patterns which differed from those of SFG rickettsiae. When several strains of each species were probed in this way, in every case except for strains of *R. conorii* identical intraspecies patterns were obtained. However, it should be noted that strains of *R. prowazekii* and *R. typhi* species tested here were earlier shown to differ in their genome structure (Artemiev *et al.*, 1991). This shows that genome regions homologous to the used probes are conserved in the members of the same species, but are not in different species. However, the resolving power of the probes was different. Probes PBH11, PBH13 and MW264 did not distinguish *R. sibirica* from *R. conorii.* However, slight difference between these species was found by MW218. These data confirm that some species of SFG, including *R. sibirica* and *R. conorii,* are very close genetically (Ralph *et al.*, 1990; Fuerst *et al.*, 1990; Regnery *et al.*, 1991). Probes PBH11 and MW218 were most suitable for discrimination of the examined species of TG and SFG. In addition, probe MW218 showed differences between the tested strains of *R. conorii.*

The presented data show that the used probes specific for DNA of the TG and SFG members of genus Rickettsia, and may be utilized for typing of these rickettsiae. The obtained hybridization patterns may be considered as criteria for genotypic identification of TG species. Unfortunately we have did not manage to assay some more of the recognized SFG species, because they were not available for us. Nevertheless, we accepted these criteria for genotyping the species occuring in the territory of the former USSR. Some of the DNA probes described in the present work were used for the characterization of an SFG isolate with unclear immunological properties from Crimea. The obtained hybridization patterns have allowed us to classify it as a novel genotype for the region (Balayeva *et al.*, 1993). It was subsequently identified as similar to *R. slovaca* (Beati *et al.*, 1992).

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