DETERMINATION OF GENOME SIZE AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM OF FOUR CHINESE RICKETTSIAL ISOLATES BY PULSED-FIELD GEL ELECTROPHORESIS

J.Z. ZHANG*, M.Y. FAN

Rickettsial Laboratory, Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine, Beijing 102206, P.R. China

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Summary. – Pulsed-field gel electrophoresis (PFGE) was used to determine the genome size and the restriction fragment length polymorphism (RFLP) of four new Chinese isolates of spotted fever group (SFG) rickettsiae. The genome size of the isolates Ha-91, BJ-90, 054 and 036 was 1253 kb, 1236 kb, 1272 kb, and 1272 kb, respectively. The isolates 054 and 036 had identical RFLP profiles. All the isolates differed in the properties under study from the so far known SFG rickettsiae. The unique RFLP profiles of the isolates supported our opinion that they are new strains of SFG rickettsiae or even new species of SFG rickettsiae.

Key words: rickettsiae; spotted fever group; Chinese isolates; pulsed-field gel electrophoresis

Introduction

SFG rickettsiae are tick-borne obligate intracellular Gram-negative bacteria. The number of new SFG rickettsial species recognized worldwide has increased rapidly in recent years due to the development of novel methods of isolation and identification (Marrero and Raoult, 1989; Raoult and Roux, 1997). To date there are evidenced more than 20 SFG rickettsial species. As this number steadily increases, the differentiation, classification and taxonomy of SFG rickettsial species appears to be very important. Traditionally, the differentiation of SFG rickettsial species has been carried out on the basis of geographical (Kreig and Holt, 1984) and serological criteria (Plotz *et al.*, 1964; Philip *et al.*, 1978). Recently, phylogenetic analysis based on comparison of selected gene sequences and antigenic similarities among rickettsial species have provided new approaches to the taxonomy of rickettsiae (Fournier *et al.*, 1998; Roux and Raoult, 1995; Roux *et al.*, 1997; Stothard and Fuerst, 1995; Woese, 1987; Weisburg *et al.*, 1989, 1991; Xu and Raoult, 1997). However, these approaches have led researchers to study only a small part of the genome of rickettsiae. On the other hand, the PFGE of DNA, a modification of conventional agarose gel electrophoresis, allows to resolve large fragments of rickettsial DNA, prepared by a low frequency restriction endonuclease digestion, by gel electrophoresis in electric field repeatedly changing its orientation (Chu *et al.*, 1986).

Owing to omission of extraction of rickettsial DNA and manipulation of agarose blocks containing rickettsial DNA a high reproducibility in preparation of DNA digestion fragments is ensured. A few DNA fragments can be easily differentiated and the size of cleaved fragments and the profile of genomic DNA after a low frequency restriction endonuclease digestion can be readily determined by comparison with standards. PFGE has been applied to the study of genomic DNA of many bacteria (Allardet-Servent

*Present address: Department of Pathology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0609, USA. E-mail: jzzhang@utmb.edu; fax: +1409-7472415.

Abbreviations: PFGE = pulsed-field gel electrophoresis; FBS = fetal bovine serum; RFLP = restriction fragment length polymorphism; SFG = spotted fever group

et al., 1988; Brosch *et al.*, 1991; Birkelund and Stephens, 1992; Ferdows and Babour, 1989; Frutos *et al.*, 1989; Kingsbury, 1969; Kauc *et al.*, 1989; McClelland *et al.*, 1987; Pyle *et al.*, 1988; Romling and Tummler, 1991; Schwartz and Cantor, 1984; Smith *et al.*, 1987).

In China, 20 strains of tick-associated rickettsiae have been isolated from patients, animals and ticks. These isolates have been identified as *R. sibirica* or new species of SFG rickettsiae (Jin *et al.*, 1993; Lou *et al.*, 1985; Wu *et al.*, 1998b; Yu *et al.*, 1991, 1993; Zhang *et al.*, 1996). In this study we used PFGE to confirm the initial identification of the Chinese isolates.

Materials and Methods

Rickettsial isolates. "*Rickettsia heilongjiangii*" isolates 054 and 036 originated from *Dermacentor silvarumin* and *Haemaphysalis concinna*, respectively (Lou *et al.*, 1985). The isolate BJ-90 originated from *D. sinicus* (Yu *et al.*, 1991) and the isolate "*R. mongolotimonae*" Ha-91 from *Hyalomma asiaticum* (Jin *et al.*, 1993). These isolates were maintained in the authors' laboratory. *R. sibirica* strain 232 and *R. japonica* strain YM were obtained from the American Type Culture Collection and D.H.Walker, University of Texas Medical Branch, Galveston, TX, USA, respectively.

Propagation of rickettsiae. All the rickettsiae were cultivated in Vero cells clone 929 at 32°C using Eagle's Minimal Essential Medium (Eurobio, France) containing 4% of fetal bovine serum (FBS; Gibco BRL, LifeTechnologies) and 2 mmol/l L-glutamine (Gibco BRL).

Rickettsial infection was monitored by Gimenez staining (Gimenez, 1964). The infected cells were harvested 6–9 days post inoculation (p.i.) when 100% of cells were infected with rickettsiae. The cells were centrifuged at 7,000 × g for 10 mins. The pellet was resuspended in the K36 buffer (16.5mmol/l KH_2PO_4, 33.5 mmol/l K_2HPO_4, 100 mmol/l KCl, and 15.5 mmol/l NaCl) and sonicated at 40 W for 2 x 10 secs on ice. The suspension was loaded onto a cushion of 30% sucrose in K36 buffer and centrifuged at 7,000 × g for 30 mins. The resulting pellet was purified by renografin density gradient centrifugation (Weiss *et al.*, 1975). The light band was harvested, washed with K36 buffer by centrifugation at 7,000 × g for 10 mins and resuspended in K36 buffer. The light band was used for PFGE.

PFGE. Agarose blocks containing rickettsial DNA were prepared and digested with proteinase K and restriction enzymes according to a method described earlier (Chu *et al.*, 1986). Three restriction endonucleases were used, namely *Sma*I, *Bss*HII (Gibco BRL and Life Technologies) and *Bst*I (Promega). A contour-clamped homogeneous electric field system (CHEF-DRII; Bio-Rad Laboratories) was used for PFGE (Chu *et al.*, 1986). Agarose gels (1% or 0.6%) were prepared from an ultrapure agarose (BRL, Sigma) in the 0.5 x TBE buffer (44.5 mmol/l Tris-HCl, 44.5mmol/l boric acid, and 1 mmol/l EDTA pH 8). Agarose blocks containing digested DNA were electrophoresed on a 1% or 0.6% agarose gel in 0.5 x TBE buffer at 14°C. Three different DNA size markers were used: the Low-Range PFG Marker (0.15 to 194 kbp), the Lambda Ladder PFG Marker (48.5 to 10,818 kbp), and the Yeast Chromosome PFG Marker (225 to 1900 kbp) (New England BioLabs) Three conditions depending on DNA size were used: 190 V with 3 to 10 sec pulses for 19 hrs, 150 V with 5 to 120 sec pulses for 33 hrs, and 180 V with 5 to 20 sec pulses for 35 hrs. The gels were stained with 0.5% ethidium bromide for 30 mins and analyzed by the Imager TM Documentation System (Apparatus, France).

The Imager System and the QGEL-1D program (APPLIGENE, France) were used to estimate the size and number of DNA fragments. The size of restriction fragments of rickettsial isolates was determined by comparison with the DNA size markers. At least three independent digestions were performed with each restriction endonuclease for each isolate. The length of an individual restriction fragment was calculated as the mean from three independent experiments.

Results and Discussion

RFLP profile of the isolates

The RFLP profiles of the genomic DNAs of four Chinese isolates, *R. sibirica*, and *R. japonica* digested with three restriction endonucleases are presented in Figs. 1–3. *Bss*HII generated six fragments for the isolates 054 and 036, three fragments for the isolate BJ-90, five fragments for *R. sibirica* and *R. japonica*, and four fragments for the isolate Ha-91. The DNA fragments ranged from 48.5 to 242.5 kbp (Fig. 1). *Sma*I produced 20 to 30 fragments of 9.42 to 145.5 kbp. There were 24 fragments for the isolates 054 and 036, 25 fragments for the BJ-90 isolate, 26 fragments for *R. sibirica*, 27 fragments for *R. japonica*, and 20 fragments for the Ha-91 isolate. *Bst*ZI produced approximately 15 to 20 fragments of 8 to 330 kbp, 12 fragments for the isolates 054 and 036, 17 fragments for the isolate BJ-90, 17 fragments for *R. sibirica*, 16 fragments for *R. japonica*, and 17 fragments for the Ha-91 isolate. *Sma*I and *Bst*ZI produced several fragments of the same mobility so that it was impossible to separate them under standard conditions. A successful separation and visualization of these fragments was achieved by use of different pulsed field conditions and gel concentrations. We found out that whereas the electrophoresis extended to 33 hrs at ramped pulse time of 5–120 secs and 0.6% agarose gel provided the best separation of large fragments, the ramped pulse time of 3–5 secs for 19 or 25 hrs and 1% agarose gel provided the best separation of small fragments. The isolates 054 and 036 had identical restriction profiles with all the three restriction endonucleases, but they differed from the isolates BJ-90 and Ha-91, *R. sibirica*, and *R. japonica* analyzed in this study and other SFG rickettsiae reported earlier (Balayeva *et al.*, 1993; Eremeeva *et al.*, 1993a, 1994, 1995; Roux *et al.*, 1992; Roux and Raoult, 1993). The restriction profiles of the

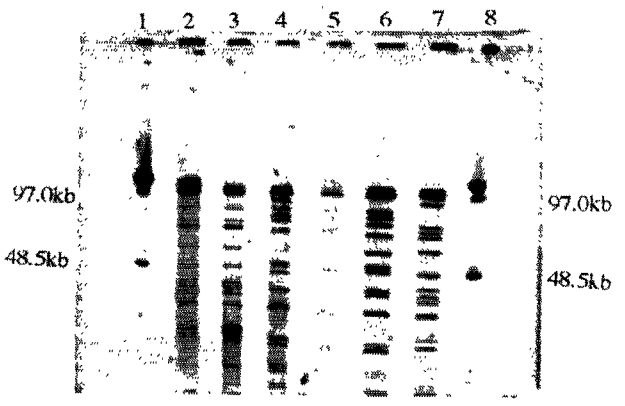

Fig. 1
PFGE of *Sma*I restriction fragments of rickettsial genomes

Lambda DNA size marker, ladder (242.5 kbp, 194.0 kbp, 145.5 kbp, 97.0 kbp, and 48.5 kbp) (lane 1); *R. heilongjiangii* strain 054 (lanes 2 and 3); isolate BJ-90 (lane 4); *R. mongolotimonae* isolate Ha-91.6 (lane 5); *R. sibirica* strain 232 (lane 6); *R. japonica* strain YM (lane 7); low-range DNA size marker (194.0 kbp, 145.5 kbp, 97.0 kbp, 48.5 kbp, 23.1 kbp, 9.42 kbp, 6.55 kbp, and 4.36 kbp) (lane 8). PGE was carried out at 200 V with ramped pulse times of 1 to 3 secs for 8 hrs and with ramped pulse times of 1 to 5 secs for 9 hrs in 1% agarose in 0.5 x TBE at 14°C.

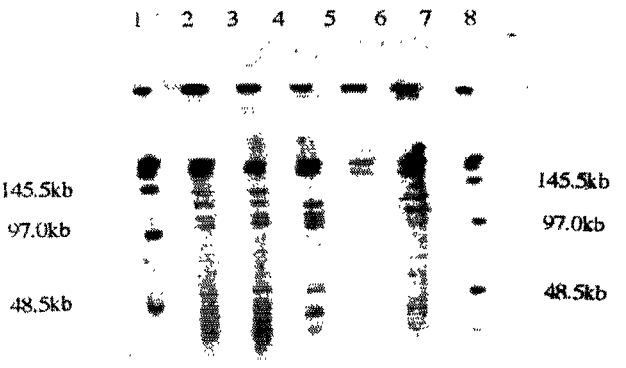

Fig. 2
PFGE of *Bst*ZI restriction fragments of rickettsial genomes

PFGE was carried out at 200 V with ramped pulse times of 3 to 10 secs for 27 hrs in 1 % agarose in 0.5 x TBE at 14°C. For the rest of the legend see Fig. 1.

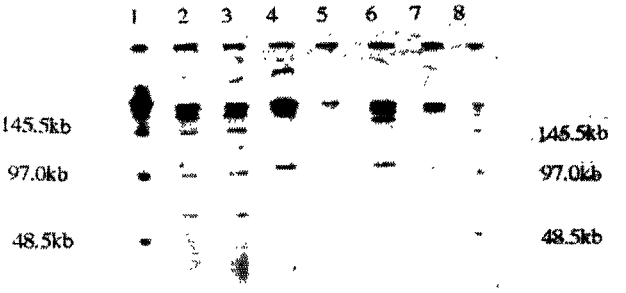

Fig. 3
PFGE of *Bss*HII restriction fragments of rickettsial genomes

PFGE was carried out at 190 V with ramped pulse times of 3 to 10 secs for 24 hrs in 1% agarose in 0.5 x TBE at 14°C. For the rest of the legend see Fig. 1

isolates BJ-90 and Ha-91 were different not only from each other but also from other SFG rickettsiae.

Genome size of the isolates

With all the restriction endonucleases used we obtained consistent values of genome size for all the four isolates, namely 1.236 kbp for the isolate Ha-91 and 1.272 kbp for the isolates 054, 036, and BJ-90. The genome size of *R. sibirica* and *R. japonica* was in accordance with the values reported earlier (Balayeva *et al.*, 1993; Eremeeva *et al.*, 1993a, 1994, 1995; Roux *et al.*, 1992; Roux and Raoult, 1993). The genome size values for the isolates, calculated from fragments obtained by digestion with the restriction endonucleases used, are listed in Table 1.

PFGE has been used to determine the molecular mass values of the genome of several species of *Rickettsia* (Eremeeva *et al.*, 1993b, 1995; Roux *et al.*, 1992; Roux and Raoult, 1993) and *Ehrlichia* (Rydkina *et al.*, 1999). Of agents causing typhus and spotted fever a high degree of intraspecies homogeneity has been demonstrated for *R. typhi* (Eremeeva *et al.*, 1993b), *R. akari* (Eremeeva *et al.*, 1995), *R. conorii* (Eremeeva *et al.*, 1993a, Roux and Raoult, 1993), *R. sibirica* (Balayeva *et al.*, 1993), *R. slovaca* (Eremeeva *et al.*, 1993a) and Astrakhan fever rickettsiae (Eremeeva *et al.*, 1994). Our results indicate that the genome size values of the four Chinese isolates under study are similar to those of other SFG rickettsiae (Eremeeva *et al.*, 1995; Roux *et al.*, 1992;

Table 1. Genome size estimated from restriction fragments obtained by digestion of genome with *Bss*HII, *Sma*I and *Bst*ZI and separation by PFGE

Rickettsia	Genome size (kbp) estimated from fragments obtained with			
	*Bss*HII	*Sma*I	*Bst*ZI	Mean size (kbp)
R. sibirica (strain 232)	1258	1238	1233	1243
R. japonica (strain YM)	1289	1272	1268	1276
R. mongolotimonae (isolate Ha-91)	1260	1270	1230	1253
R. heilongjiangii (strain 054)	1290	1268	1258	1272
D. sinicus (isolate BJ-90)	1224	1256	1230	1236

Roux and Raoult, 1993). PFGE has been demonstrated not only as a good method for the study of the genome size of rickettsiae (Eremeeva *et al.*, 1993b, 1995; Frutos *et al.*, 1989; Gimenez *et al.*, 1990; Roux *et al.*, 1992; Roux and Raoult, 1993; Rydkina *et al.*, 1999) but also as a reliable method for the identification of rickettsial isolates at the species level (Eremeeva *et al.*, 1993b; Roux and Raoult, 1993). Comparing the restriction profiles obtained in this study with those of other SFG rickettsiae reported earlier (Balayeva *et al.*, 1993; Eremeeva *et al.*, 1993a, 1994a, 1995; Roux *et al.*, 1992; Roux and Raoult, 1993) we concluded that the four Chinese isolates differ from other SFG rickettsiae and can be classified into three groups represented by (i) the isolate Ha-91, (ii) the isolate BJ-90, and (iii) the isolates 054 and 036.

Earlier phylogenetic, genotypic and antigenic analyses have indicated the following grouping.

The isolates Ha-91 and BJ-90 were grouped in the same cluster with *R. sibirica* (Jin *et al.*, 1993; Xu and Raoult, 1997; Yu *et al.*, 1991, 1993; Zhang *et al.*, 2000b). The isolate BJ-90 was considered to be a *R. sibirica* variant since it was genotypically identical with and antigenically related to *R. sibirica*. However, its SDS-PAGE profile was different (Yu *et al.*, 1993; Zhang *et al.*, 2000b). The isolate HA-91 was considered a new species of SFG rickettsiae as it was both antigenically and genotypically unique among SFG rickettsiae (Lou *et al.*, 1985, Yu *et al.*, 1993).

In this study, we found out that not only different PFGE restriction profiles exist between the isolate Ha-91 and *R. sibirica* but also between the isolate BJ-90 and *R. sibirica*. Yu *et al.* (1993) have suggested the isolate Ha-91 as new species. Fournier *et al.* (1998) designated it *"Rickettsia mongolotimonae"*. Our data further support our opinion that the isolate Ha-91 is a new species of SFG rickettsiae. The results indicating the existence of genomic variation between the isolate BJ-90 and *R. sibirica* (strain 246) presented here are not in accordance with our results reported earlier (Zhang *et al.*, 2000) and made us reassess the taxonomic position of the isolate BJ-90. Studies of other authors have shown that a 93% nucleotide variability occurs in the form of interspecies variations (Fuemeeva and Poetter, 1991; Ralph *et al.*, 1990) and that a high degree of intraspecies homogeneity occurs between the typhus group and SFG rickettsiae. An earlier report on typing strains of different bacteria has shown that point mutations of chromosomal DNA make a little contribution only to RFLP (Fuerst *et al.*, 1990). Plasmids, insertion elements, and repetitive sequences have been found responsible for DNA rearrangements (Fuerst *et al.*, 1990; Hall, 1994; Herrmann *et al.*, 1991). However, to date, plasmids and insertion elements have not been found in SFG rickettsiae species. Repetitive sequences have been detected in the *ompA* gene of SFG rickettsiae (Gilmore and Hacstadt, 1991) and a strain-specific variability has been shown among strains of *R. rickettsii* (Gilmore and Hacstadt, 1991) and *R. conorii* (Walker *et al.*, 1995). However, earlier reports on PFGE analysis in combination with DNA probe hybridization and DNA sequence comparison have described a remarkable genomic identity among different isolates of *R. conorii* and *R. rickettsii* (Fuemeeva and Poetter, 1991; Ralph *et al.*, 1990). Considering the fact that only three genes have been followed in earlier genotypic and phylogenetic studies, PFGE provides more information on the genome, its conservation and stability within *R. sibirica* strains (Balayeva *et al.*, 1993) and SFG rickettsiae (Eremeeva *et al.*, 1993a, 1994a, 1995; Roux *et al.*, 1992; Roux and Raoult, 1993). Therefore, the taxonomic position of the isolate BJ-90 should be reconsidered.

Earlier phylogenetic, genotypic and antigenic studies have led to the opinion that the isolate 054 is related to and should be clustered together with *R. japonica* (Chen *et al.*, 1998; Zhang *et al.*, 1997, 2000a). In this study we found out that the restriction profiles of the isolate 054 are different from those of *R. japonica* and other SFG rickettsiae (Balayeva *et al.*, 1993; Eremeeva *et al.*, 1993a, 1994a, 1995; Roux *et al.*, 1992; Roux and Raoult, 1993). The isolates 054 and 036 had identical RFLP profiles but different from those of other SFG rickettsial species. Therefore, they should be considered a new species of SFG rickettsiae.

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