GENOTYPIC IDENTIFICATION OF THREE NEW STRAINS OF SPOTTED FEVER GROUP RICKETTSIAE ISOLATED IN CHINA

J.Z. ZHANG[1], M.Y. FAN[1], D.Z. BI[1], W.F. CUI[2], Y.F. HAN[2]

[1]Department of Rickettsiology, Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine, 102206 Beijing; [2]Suifenhe City Quarantine Station, Suifenhe, Heilongjiang, P.R. of China

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Summary. – Polymerase chain reaction (PCR) and restriction endonuclease fragment length polymorphism (RFLP) analysis were used to characterize the genotypic diversity of three isolates of spotted fever group (SFG) rickettsiae isolated from ticks in China. A primer pair designed from DNA sequence encoding 190 K protein antigen of *R. rickettsii* and genomic DNAs obtained from the isolates were used in PCR. The PCR products were cleaved with restriction endonucleases *Pst*I and *Rsa*I, and the digestion patterns were analyzed by polyacrylamide gel electrophoresis (PAGE) and compared with those of all known species and strains of SFG rickettsiae. The results showed that three isolates had the same PCR products as the other SFG rickettsiae under comparison. HL-93 strain, isolated from *Hemophysalis concinna* ticks collected in Hulin County, Heilongjiang Province, had unique *Pst*I digestion pattern among SFG rickettsiae; strains BJ-93 and 053, isolated from *Dermacentor sinicus* and *Haemaphysalis concinna* ticks collected in Changping County, Beijing City, and Suifenhe City, Heilongjiang Province, respectively, had the same *Pst*I and *Rsa*I digestion patterns as strains *R. sibirica* 246, BJ-90 and IMTO-85. The present study demonstrated that the BJ-93 and 053 strains were genotypically identical with *R. sibirica* and the HL-93 strain was genotypically unique among SFG rickettsiae.

Key words: spotted fever group rickettsiae; Chinese isolates; genotyping; restriction endonuclease fragment length polymorphism; polymerase chain reaction

Introduction

SFG rickettsiae occur worldwide (Weiss *et al.,* 1984). A serological survey made by use of complement fixation test in Abagnar Qi, Inner Mongolia, in 1958 first demonstrated antibodies in 17 of 154 (11%) healthy persons against *R. sibirica* antigen, and in 41of 154 (26.6%) healthy persons with *R. akari* antigen (Fan *et al.,* 1964). Recently, SFG rickettsioses were recognized also in P.R. China. Although *R. sibirica* was considered the only causative agent of Chinese SFG rickettsiosis-North Asian tick-borne spotted fever (Cheng *et al.,* 1989; Fan *et al.,* 1988), at least four isolates of SFG rickettsiae were obtained in P.R. China (Yu *et al.,* 1993; Lou *et al.,* 1985). In 1993, two rickettsial strains were isolated from *Haemaphysalis concinna* and *Dermacentor sinicus* ticks, collected in Hulin County, Heilongjiang Province, and Changping County, Beijing City, in P.R. China. The two isolates were identified as members of SFG rickettsiae by the methods based on immunofluorescence, morphology, PAGE in the presence of SDS, and Western blot analysis with polyclonal and monoclonal antibodies (Zhang *et al.,* 1995); strain HL-93 was identified as a new member of SFG rickettsiae. In order to further characterize the two isolates, PCR and RFLP described by Regnery *et al.* (1991) were employed to determine their genotype. In this paper we describe the genotype of the two isolates and also of previously unknown rickettsial strain 053.

Abbreviations: PAGE = polyacrylamide gel electrophoresis; PCR = polymerase chain reaction; RFLP = restriction endonuclease fragment length polymorphism; SDS = sodium dodecyl sulphate; SFG = spotted fever group;

Materials and Methods

Rickettsial strains HL-93 and BJ-93 were isolated from *Haemaphysalis concinna* and *Dermacentor sinicus* ticks, respectively, collected in Hulin County, Heilongliang Province, and Changping County, Beijing City, P.R. China. Strain 053 was isolated from *Haemaphysalis concinna* ticks from Suifenhe City, Heilongjiang Province, in 1985. The following rickettsial reference strains were used: *R. akari* strain Kaplan was obtained from American Type Culture Collection; *R. australis* strain W58 and *R. parkeri* were obtained from Department of Health Laboratory, Columbus, OH, USA; *R. sibirica* strain 246, *R. rickettsii* strain R, *R. conorii* strains Barbash and Simko were provided by the Chinese Military Academy for Medical Research; *R. conorii* strain Malish 7 was obtained from The University of Texas Medical Branch, Galveston, USA; *R. conorii* strain India was provided by Gamaleya Research Institute of Epidemiology and Microbiology, Moscow, Russia; *R. sibirica* strains BJ-90, IMTO-85 and JH-74 were provided by the Department of Rickettsiology, Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine, Beijing, P.R. China; strain HA-91 was obtained from Inner Mongolia Anti-epidemic Station, Huhehot, Inner, Mongolia, P.R. China.

Cultivation, purification and DNA extraction. All the employed rickettsial strains were cultivated in specific pathogen-free chicken embryo yolk sacs as described by Stoenner *et al.* (1962), purified according to Hanson *et al.* (1981), and their DNA extracted by the method of Regnery *et al.* (1991).

PCR amplification, DNA digestion and PAGE. A pair of oligonucleotide primers (Rr190.70p: ATGGCGAA-TATTTCTCCAAAA; Rr190.602p: AGTGCAGCAT-TCGCTCCCCCT) used in PCR was derived from DNA sequence of a gene encoding the 190 K antigen of *R. rickettsii*. It was provided by the Chinese Academy of Medicine, Beijing. The amplification was carried out in 50 µl volume in Gene ATAQ Controller (Pharmacia) in 30 cycles (95°C for 40 secs, 48°C for 40 secs, 66°C for 80 secs, the last cycle extended to 5 mins). Non-infected yolk sac was used as a negative control. The presence of amplified PCR products was confirmed by electrophoresing 10 µl of each mixture in 1.2% agarose gel. *Hinf* I-digested pBR322 DNA was employed as size marker. PCR products were digested with *Pst* I and *Rsa* I at 37 °C for 3 hrs in a volume of 20 µl, separated in vertical 8% polyacrylamide gel run at 7 V/cm for 4 hrs, and visualized under UV light after staining with ethidium bromide. *Hinf* I-digested pBR322 DNA was employed as a size marker.

Results

PCR amplification of DNA

The nucleotide primer pair, Rr190.70p and Rr190.602p, primed the synthesis of DNA using DNAs extracted from three isolates and rickettsial reference strains as templates. The PCR-amplification products were visualized after aga-

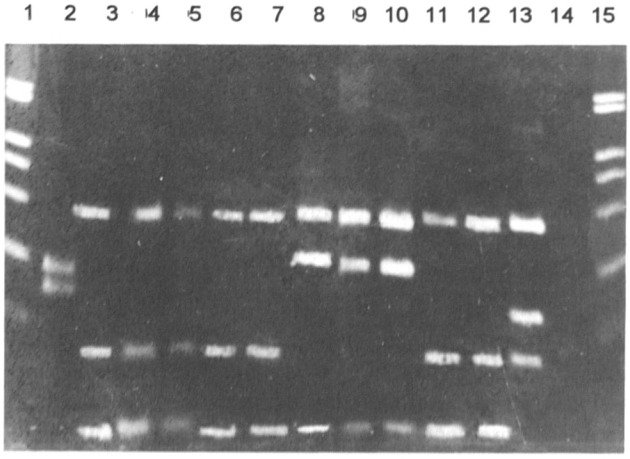

Fig. 1
PCR/RFLP analysis of various SFG rickettsial strains with *Pst*I
Lanes 1,15: *Hinf*I-digested pBR322 as size marker (1631, 517, 506, 396, 344, 298, 221+220, 154, and 75 bp); lane 2: *R. conorii* strain Barbash; lane 3: *R. sibirica* strain BJ-90; lane 4: *R. sibirica* strain 246; lane 5: *R. innermonglii* strain Ha-91; lane 6: *R. parkeri*; lane 7: *R. sibirica* strain IMTO-85; lane 8: *R. conorii* strain Malish 7; lane 9: *R. conorii* strain India; lane 10: *R. rickettsii* strain R; lane 11: strain 053; lane 12: strain BJ-93; lane 13: strain HL-93; Lane 14: negative control

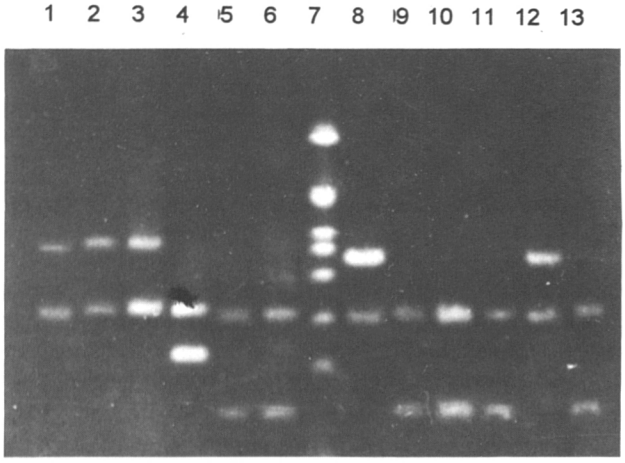

Fig. 2
PCR/RFLP analysis of various SFG rickettsial strains with *Rsa*I
Lane 1: *R. parkeri;* lane 2: *R conorii* strain Malish 7; lane 3: *R. conorii* strain India; lane 4: *R. conorii* strain Barbash; lane 5: *R. rickettsii* strain R; lane 6: *R. sibirica* strain 246; lane 7: *Hinf*-digested pBR322 as size marker (see Fig. 1); lane 8: *R. innermonglii* strain Ha-91; lane 9: *R. sibirica* strain BJ-90; lane 10: strain BJ-93; lane 11: strain 053; lane 12: strain HL-93; lane 13: *R. sibirica* strain IMTO-85

rose gel elestrophoresis. The results showed that the DNA fragments were amplified from all the employed rickettsial strains except *R. akari* and *R. australis* (data not shown). The size of all the PCR amplification products was approximately 533 bp except *R. conorii* strain Barbash (approximately 427 bp). No products was amplified from non-infected yolk sac DNA.

PCR-RFLP

The amplified products of all rickettsial strains tested were digested with restriction endonucleases *Pst*I and *Rsa*I, respectively. The *Pst*I digestion showed that all tested strains could be divided into four distinct genotype groups: The first group, including strains BJ-93, BJ-90, *R. sibirica* strain 246, *R. parkeri,* strains IMTO-85, 053 and Ha-91, had the same PCR-RFLP pattern. The second group, including *R. rickettsii* strain R, *R. conorii* strains Malish 7 and India had identical pattern. The third and fourth groups include *R. conorii* strain Barbash and strain HL-93, respectively. The two latter strains had patterns different not only from each other but also from those of other tested strains (Fig. 1). The *Rsa*I digestion of amplified products of rickettsial strains led to three groups (Fig. 2). The first group including, strain HL-93, *R. parkeri,* strain Ha-91, *R. conorii* strains Malish 7 and India. The second, including strains BJ-93, BJ-90, IMTO-85 and 053, *R. sibirica* strain 246 and *R. rickettsii*. *R. conorii* strain Barbash had an unique pattern (third group).

Discussion

SFG rickettsiae are obligate intracellular parasitic microorganisms. Because they share certain common features of ecologic interest and live free in the cytoplasm of acarine arthropod or verterbrata cells, their isolation and identification are rather difficult. They were classified according to geographical criteria (Weiss *et al.*, 1984), serological techniques (Plotz *et al.*, 1964; Bell *et al.*, 1960; Philip *et al.*, 1978), SDS-PAGE (Pederson *et al.*, 1978; Li *et al.*, 1988) and Western blot analysis (Beati *et al.*, 1989). But all the identification and classification techniques mentioned above have both advantages and disadvantages.

Nucleotide analysis of the genome generally and of specific genes particularly has been used to identify SFG rickettsiae (Anderson *et al.*, 1989). Also, analysis of restriction endonuclease digests of DNA (Regnery *et al.*, 1983), hybridization with isotope-labelled, cloned DNA probes (Fuerst *et al.*, 1990; Regnery *et al.*, 1985), cross-hybridization of genomic DNA (Myers *et al.*, 1980) and analysis of 16 S rRNA sequences (Weisburg *et al.*, 1898) were employed. These methods provided the best basis for determination of genetic relationships between specific genes among various species, but they turned out to be not practical and suitable for routine identification of various isolates of SFG rickettsiae, as their genetic variability is lower than that found within a single species of enteric bacteria (Fuerst *et al.*, 1991; Ralph *et al.*, 1990).

In 1991, Regnery and coworkers (Regnery *et al.*, 1991) described the genotypic identification of rickettsiae by PCR-RFLP analysis. This technique has the advantage that cultivation and purification steps are not necessary, it requires just a very small amount of live or killed rickettsiae, and can differentiate rickettsial species and genotypes as well as estimate genetic divergence among selected genes. Thus, this technique has been employed to identify rickettsiae in ticks (Gage *et al.*, 1994; Beati *et al.*, 1994; Bacellar *et al.*, 1995), and new isolates (Beati *et al.*, 1992; Yan *et al.*, 1993; Yu *et al.*, 1993).

In order to further characterize the three isolates, their DNA was amplified with the primer pair Rr190.70p and Rr190.602, designed for *R. rickettsii* 190 K antigen gene, the PCR products were digested with endonucleases *Pst*I and *Rsa*I, and electrophoresed in PAGE. Our results showed that the three isolates had the same PCR amplification pattern as other tested SFG rickettsiae except *R. australis* and *R. akari*, which yielded no PCR product in accord with the results of Regnery *et al.* (1991) and Yan *et al.* (1993). Comparing the PCR-RFLP patterns of HL-93 strain with those of other SFG rickettsial strains, obtained in this study or published previously (Beati *et al.*, 1992, 1994; Regnery *et al.*, 1991; Yan *et al.*, 1993; Yu *et al.*, 1993), we found that the *Pst*I pattern was unique among SFG rickettsiae and the *Rsa*I pattern was identical with those of *R. parkeri,* strain Ha-91, *R. conorii* strains Malish 7 and India, but different from those of other Chinese isolates (BJ-93, BJ-90, IMTO-85 and 053) and *R. sibirica* strain 246. Strains BJ-93 and 053, isolated from *D. sinicus* and *H. concinna* ticks, had the same *Pst*I and *Rsa*I patterns as *R. sibirica* strain 246, BJ-90 and IMTO-85. The results on SFG rickettsiae obtained in this study are in accord with those of previous studies (Regnery *et al.*, 1991; Beati *et al.*, 1992, 1994; Yan *et al.*, 1993; Yu *et al.*, 1993). This fact supports the reliability of our results on the PCR-RFLP patterns of the three isolates.

The present study demonstrated that strains BJ-93 and 053 are genotypically identical with *R. sibirica*, and strain HL-93 is genotypically unique among SFG rickettsiae. We think that strain HL-93 should be considered a new member of SFG rickettsiae on the basis of the data presented here and published previously (Zhang *et al.*, 1995, 1996).

In 1993, Yu *et al.* (1993) suggested that there are three types of SFG rickettsiae occuring in northern China; type 1, antigenically and genotypically identical with *R. sibirica*, represented by strain IMTO-85; type 2, antigenically related to and genotypically identical with *R. sibirica,* but with SDS-PAGE profile different from those of other rickettsial strains;

type 3, both antigenically and genotypically unique among SFG rickettsiae, represented by strain Ha-91.

Our study not only supports this categorization of different types of SFG rickettsiae, but it also identified one new member of SFG rickettsiae.

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