SEQUENCE ANALYSIS AND COMPARISON OF 190 K SURFACE ANTIGEN GENE FRAGMENT OF A NEW SPECIES OF SPOTTED FEVER GROUP RICKETTSIAE

J.Z. ZHANG, M.Y. FAN, D.Z. BI

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

Received July 11, 1996; revised September 19, 1996

Summary. – A 533 bp long PCR product amplified from rickettsial strain HL-93 DNA with the primer pair Rr 190.70p and Rr 190.602n, designed from DNA sequence encoding 190 K protein antigen of *R. rickettsii,* was cloned into plasmid vector PGEM-T and sequenced. The primer-flanking region of the product, an open reading frame, was 491 bp long. The sequence of the product was compared with those of the corresponding regions of DNAs of *R. rickettsii* (strain R), *R. japonica* (strain VR1363) and *R. conorii* (strain Malish 7) which were reported earlier by other authors. The results showed that 23, 31 and 52 nucleotides in the compared sequence in strain HL-93 differed from those in *R. japonica, R. rickettsii* and *R. conorii,* respectively. The homologies of strain HL-93 with *R. japonica, R. rickettsii* and *R. conorii* were 95.6%, 94% and 90% in nucleotide, and 89%, 87% and 80% in putative amino acid sequences. We consider strain HL-93 as a new member of spotted fever group (SFG) rickettsiae on the basis of a high degree of homology and genetic divergence in the nucleotide sequence of a part of the 190 K protein gene.

Key words: rickettsiae; spotted fever group; strain HL-93; 190 K protein; nucleotide sequence; PCR

Introduction

Rickettsial strain HL-93 was isolated from *Haemaphysalis concinna* ticks in Hulin County of Heilongjiang Province in P.R. of China. It was identified as a new member of SFG rickettsiae by the methods of standard serology, morphology, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot analysis with polyclonal and monoclonal antibodies, PCR and PCR followed by restriction fragment length polymorphism (PCR/RFLP) analysis (Zhang *et al.,* 1996). In order to further classify rickettsial strain HL-93, the PCR product, amplified from its DNA with the primer pair Rr 190.70p and Rr 190.602n, designed from DNA sequence encoding the 190 K protein antigen of *R. rickettsii,* was cloned and sequenced. In this paper, we report the nucleotide sequence of the PCR product (a part of the 190 K protein gene) amplified from rickettsial strain HL-93 DNA and compare it with those of the corresponding region of DNAs of *R. japonica, R. rickettsii* and *R. conorii.*

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

Materials and Methods

Rickettsial strain HL-93 was isolated from *Haemophysalis concinna* ticks in Hulin County, Heilongjiang Province, P.R. of China, in 1993.

Cultivation, purification and DNA extraction. Cultivation and purification of rickettsial organism were performed as described by Stoenner *et al.* (1962) and Hanson *et al.* (1981). Rickettsial DNA was extracted according to Regnery *et al.* (1991).

PCR amplification was carried out using a pair of *R. rickettsii* 190 K antigen gene primers, Rr 190.70p and Rr 190.602n, de-

scribed by Regnery *et al.* (1991). 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). PCR assay included a negative control (no template DNA), a positive control (*R. sibirica* strain 246 DNA) and control material from non-infected yolk sac. 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.

Cloning. PCR amplified product to be sequenced was cloned into plasmid vector PEGM-T (Promega). *E. coli* strain JM101 cells were transformed by the method described by Sambrook *et al.* (1989). The transformants carrying the vector with the DNA insert in both directions were screened on colour plates containing X-Gal, IPTG and Ampicillin, and the vector recombinants were identified by the methods of PCR amplification, restriction endonuclease digestion and dot hybridization with an oligonucleotide probe.

DNA sequencing of the PCR product was performed by the dideoxy method according to the manufacturer's instruction using a chemiluminescent DNA sequencing kit (Promega). Sequenase™ ver.2.0 (United States Biochemical Co., Cleveland, OH, USA) was employed. DNA fragments were separated in Sequigen Nucleic Acid Sequencing Cell (BioRad Laboratories).

Results

PCR amplification of rickettsial strain HL-93 DNA

The nucleotide primer pair Rr 190.70p and Rr 190.602n were employed (Regnery *et al.*, 1993). DNAs from strain HL-93, strain Breinl of *R. prowazekii*, strain 246 of *R. sibirica* and *E. coli* JM101 cells were used as templates. The PCR products were separated by 1.5% agarose gel electrophoresis. The results showed that DNA fragments of expected size (approximately 533 bp) were amplified from strain HL-93 and strain 246 of *R. sibirica* but no products were amplified from strain Breinl of *R. prowazekii* and *E. coli* JM101 cells (data not shown).

PCR amplification of recombinants

The genomic DNAs from PGEM-T vector recombinants, strain HL-93 and *E. coli* JM101 cells served as templates. PCR amplifications were carried out using the primer pair Rr 190.70p and Rr 190.602n. The results showed that 533 bp long DNA fragments were amplified from template DNAs from strain HL-93 and vector recombinants. Original PGEM-T vector and *E. coli* JM101 cell genomic DNAs did not yield any PCR products (data not shown).

Restriction endonuclease digestion of recombinants

Recombinants were digested with two combinations of restriction endonucleases, *Nco*I + *Sal and Pst*I + *Sph*I, and analyzed by PAGE. The results confirmed that a fragment of the strain HL-93 190 K antigen gene had been cloned into the vector (data not shown).

Dot hybridization of vector recombinants with oligonucleotide probe

The PCR product of strain HL-93 was labelled with digoxin via random hexanucleotide primers and Klenow enzyme, and subsequently used as a probe for dot hybridization. The results showed that DNAs of the vector recombinants and strain HL-93 reacted with the probe (data not shown).

DNA sequence of the 190 K antigen gene fragment of strain HL-93 and its comparison with those of R. japonica, R. rickettsii and R. conorii

The DNA sequence of the 190 K antigen gene fragment of strain HL-93 is shown in Fig. 1. In the present study, we were able to detect up to 300 nucleotides in one run without difficulty. The sequencing data from regions of opposite directions showed an overlapping of at least 30 nucleotides. The complete sequence of the 190 K antigen gene fragment (533 bp) of strain HL-93 was determined for both strands.

The primers used by us in this study have been known to amplify 533 bp DNA fragments of *R. japonica, R. rickettsii* and *R. conorii* 190 K antigen genes (Yan *et al.*, 1994; Anderson *et al.*, 1990; Patricia *et al.*, 1994). In the present study, the sequence of 533 bp, an open reading frame, was also detected in the PCR product amplified from strain HL-93 DNA using the same primer pair. The primer-flanking region contained 491 nucleotides. The overall nucleotide sequence of the PCR product amplified from 190 K antigen gene of strain HL-93 was compared with those of the corresponding regions of *R. japonica* (Yan *et al.*, 1994), *R. rickettsii* (Anderson *et al.*, 1990) and *R. conorii* (Patricia *et al.*, 1994) 190 K antigen genes. The results showed that 31 nucleotide substitutions were found in *R. rickettsii*, 23 in *R. japonica* and 52 in *R. conorii* (Fig. 1). The genetic homologies of strain HL-93 with *R. japonica, R. rickettsii* and *R. conorii* in the compared 533 bp sequence were 95.6% (510/533), 94% (502/533) and 90% (481/533), respectively. The nucleotide substitutions resulted in changes in 19, 22 and 34 codons in *R. japonica, R. rickettsii* and *R. conorii*, respectively (Fig. 2). Therefore, the homologies of the putative amino acid sequences of strain HL-93 with those of *R. japonica, R. rickettsii* and *R. conorii* in the compared region were 89% (158/177), 87.5% (155/177) and 80.7% (143/177), respectively. All these results suggest that a high degree of homology exists among the four species compared.

Discussion

SFG rickettsiae are obligate intracellular bacteria transmitted to humans by the bite of infected ticks. Multiple spe-

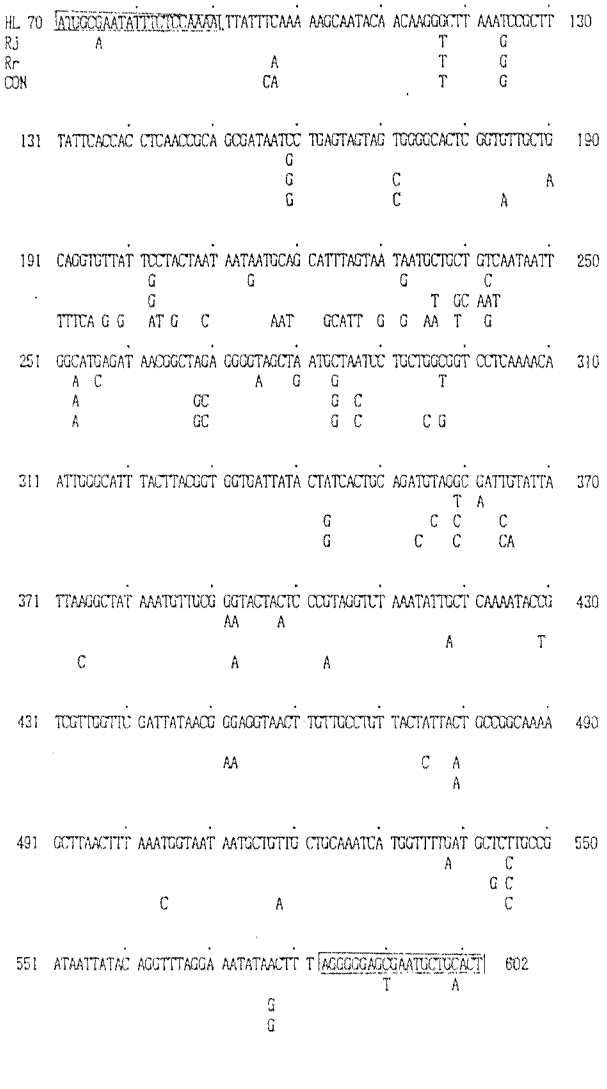

Fig. 1
Nucleotide sequence of the 533 bp PCR product of the strain HL-93 DNA

The numbering of the HL-93 (HL) sequence is aligned to that of *R. rickettsii.* The nucleotide sequences of *R. japonica* (Rj), *R. rickettsii* (Rr) and *R. conorii* (Rc) 190 K antigen genes were reported by Yan *et al.* (1994), Anderson *et al.* (1990) and Patricia *et al.* (1994), respectively. The nucleotide substitutions in the *R. japonica, R. rickettsii* and *R. conorii* sequences are shown by letters at the corresponding positions. Boxes represent the sequences of the primer portions.

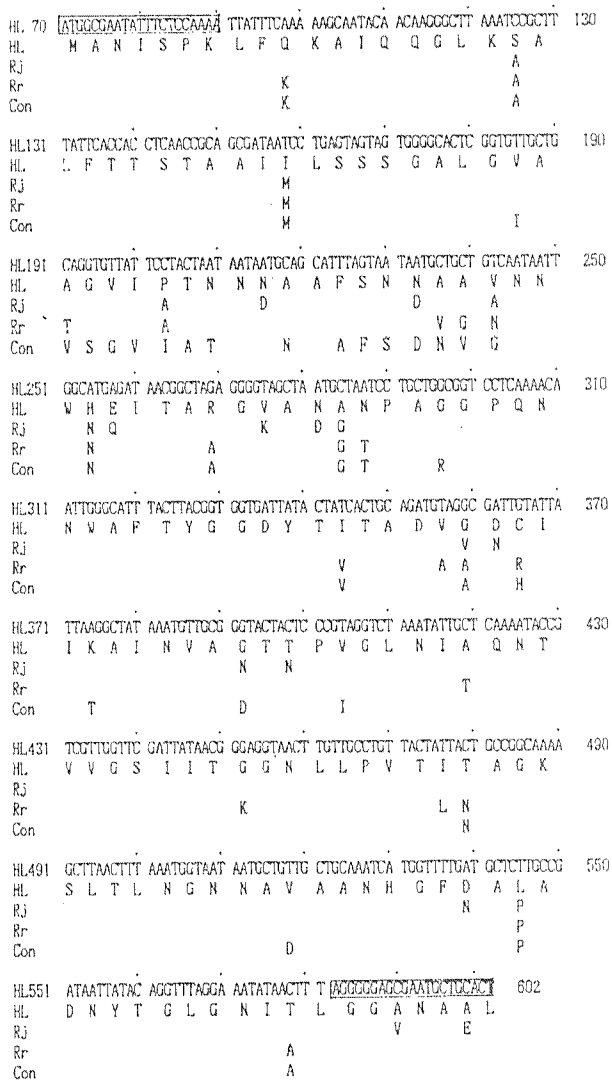

Fig. 2
Putative amino acid sequence of the 533 bp PCR product of the strain HL-93 DNA

The putative amino acid substitutions in the *R. japonica, R. rickettsii* and *R. conorii* sequences are shown by letters at the corresponding positions. For the rest of the legend see Fig. 1.

cies of SFG rickettsiae have been recognized (Weiss *et al.*, 1984; Walker *et al.*, 1989). Recently, several new SFG rickettsial serotypes have been reported (Pretzman, 1994; Stenos *et al.*, 1994). Since different SFG species may share certain common features of ecologic interest (e.g. geographic distribution, common arthropod vectors) and considerable serologic cross-reactivity (Weiss *et al.*, 1984), it is difficult to identify SFG rickettsial species. Although there are many methods currently available for identification of rickettsial species, they have technical limitations. E.g., serologic identification of rickettsial isolates is complicated by substantial cross-reactivity between recognized species (Philip *et al.*, 1978). Although polypeptide patterns of rickettsial strains have been studied for the purpose of species differentiation (Anacker *et al.*, 1984), electrophoretic polypeptide analysis is also complicated by several factors, e.g., rickettsiae to be identified must be rigorously purified, freed of contaminating host cell proteins, without a loss or modifi-

cation of important rickettsial proteins (epitopes). Mouse monoclonal antibodies have been used to differentiate selected species (McDade *et al.*, 1988), but they are not easily prepared and are not generally available. The immunoblot analysis with polyclonal mouse sera or monoclonal antibodies has been applied to analyze the antigenicity of some isolated strains (Yu *et al.*, 1993;). Serological methods as well as SDS-PAGE have some disadvantages. As genomic nucleic acid is an inheritable material of rickettsiae of considerable stability, an analysis of specific genes has been used to identify genotypic relationships among various species by some reserchers. E.g., a complete nucleotide sequence of specific genes (Anderson *et al.*, 1989), an analysis of restriction endonuclease digests of DNA (Regnery *et al.*, 1983), hybridization with isotope-labelled, cloned DNA probe (Regnery *et al.*, 1985), cross-hybridization of genomic DNA (Myers *et al.*, 1980) and analysis of ribosomal gene sequences (Weisburg *et al.*, 1989). These methods provide the best basis for determining genetic relationships among specific genes of various species but they cannot currently be considered a reasonable approach for routine identification of multiple isolates. Recently, PCR technique has been employed to detect the presence of rickettsiae in clinical specimens (Carl *et al.*, 1990), vectores (Webb *et al.*, 1990; Lange *et al.*, 1992) and rodent organs (Zhang *et al.*, 1995). On the basis of PCR technique, Regnery *et al.* (1991) has described a method that involves the use of defined rickettsial gene sequences amplified by PCR techniques as the means for non-isotopic RFLP differentiation of rickettsial species and genotypes as well as for estimating genetic divergence among selected genes. This technique has also been employed to identify rickettsiae in ticks (Beati *et al.*, 1992, 1994; Bacellar *et al.*, 1995; Gage *et al.*, 1994).

In this study, we investigated the nucleotide sequence of a genomic fragment of rickettsial strain HL-93. The PCR product amplified with the primer pair Rr 190.70p and Rr 190.602n was cloned, sequenced and compared with the corresponding region of *R. japonica, R. rickettsii* and *R. conorii* DNAs reported earlier by other authors (Anderson *et al.*, 1990; Patricia *et al.*, 1994; Yan *et al.*, 1994). Our results demonstrated that there exists a genetic divergence but still a high degree of homology in the nucleotide sequence of the 533 bp region of the 190 K antigen gene among the four compared rickettsiae. We propose that the strain HL-93 should be considered a new member of SFG rickettsiae.

Acknowledgements. We are grateful to Dr. W. J. Tan of the Institute of Virology and Prof. Y. F. Zhang of the Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine, Beijing, for their helpful comments regarding the work, and to Prof. S. R. Huang of the Institute of Tropical Medicine, Beijing, for his help in critical reviewing of the manuscript. This work was supported by The National Natural Science Foundation of P.R. of China.

References

Anacker RL, Philip RN, Williams JC, List RH, Mann RE (1984): Biochemical and immunochemical analysis of Rickettsia rickettsii strains of various degrees of virulence. *Infect. Immun.* **44**, 559–564.

Anderson BE, Tzianabos T (1989): Comparative sequence analysis of a genus-common rickettsial antigen gene. *J. Bacteriol.* **171**, 5199–5201.

Anderson BE, McDonald GA, Jones DC, Regnery RL (1990): A protective antigen of Rickettsia rickettsii has tandemly repeated, near-identical sequences. *J. Immunol.* **58**, 2760–2769.

Bacellar F, Regnery RL, Nuncio MS, Filipe AR (1995): Genotypic evaluation of rickettsial isolates recovered from various species of ticks in Portugal. *Epidemiol. Infect.* **114**, 169–178.

Beati L, Finidori J-P, Gilot B, Raoult D (1992): Comparison of microimmunofluorescence serologic typing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and polymerase chain reaction followed by restriction fragment length polymorphism analysis for identification of rickettsiae: characterization of two new rickettsial strains. *J. Clin. Microbiol.* **30**, 1922–1930.

Beati L, Humair P-F, Aeschlimann A, Daoult D (1994): Identification of spotted fever group rickettsiae isolate from Dermacentor marginatus and Ixodes ricinus ticks collected in Switzerland. *Am. J. Trop. Med. Hyg.* **51**, 138–148.

Carl M, Tibbs CW, Dobson ME, Paparello S, Dasch GA (1990): Diagnosis of acute typhus infection using the polymerase chain reaction. *Infect. Dis.* **161**, 791–793.

Gage KL, Schrumpf ME, Karstens RH, Burgdorfer W, Schwan TG (1994): DNA typing of rickettsiae in naturally infected ticks using a polymerase chain reaction restriction/fragment length polymorphism system. *Am. J. Trop. Med. Hyg.* **50**, 247–260.

Hanson BA, Wisseman CL, Waddell AJr, Silverman DJ (1981): Some characteristics of heavy and light bands of Rickettsia prowazekii on rinografin gradients. *Infect. Immun.* **34**, 596–604.

Lange JV, Dessouky AGE, Manor E, Merdan AI, Azad AF (1992): Spotted fever rickettsiae in ticks from the northern Sinai Governate, Egypt. *Am. J. Trop. Med. Hyg.* **46**, 546–551.

McDade JE, Black CM, Roumillat LF, Redus MA, Spruill CL (1988): Addition of monoclonal antibodies specific for Rickettsia akari to the rickettsial diagnostic panel. *J. Clin. Microbiol.* **26**, 2221–2223.

Myers WF, Wisseman CL (1980): Genetic relatedness among the typhus group rickettsiae. *Int. J. Syst. Bacteriol.* **30**, 143–150.

Patricia A, Valdes C, Weiss E, Walker DH (1994): Sequence analysis of the 190 kDa antigen-encoding gene of Rickettsia conorii (Malish 7). *Gene* **140**, 115–119.

Philip RN, Casper EA, Burgdorfer W, Gerloff RK, Hughes LE, Bell EJ (1978): Serologic typing of rickettsiae of the spotted fever group by indirect microimmunofluorescence. *J. Immunol.* **121**, 1961–1968.

Pretzman C, Stothard DR, Ralph D, Fuerst PA (1994): A new species of rickettsia isolated from the Lone Star tick, Am-

blyomma americanum (Ixodidae). In *Program and Abstracts of 11th Sesqui-Annual Meeting of American Society for Rickettsiology and Rickettsial Diseases.* Simons Island, GA, p. 24.

Regnery RL, Tzianabos T, Esposibos JJ, McDade JE (1983): Strain differentiation of epidemic typhus rickettsiae (Rickettsia prowazekii) by DNA restriction endonuclease analysis. *Curr. Microbiol.* **8**, 355–358.

Regnery RL, Spruil CL, Wood DO (1985): Analysis of rickettsial DNA by hybridization with cloned rickettsial DNA probes. In Kazár J (Ed.): *Rickettsiae and Rickettsial Diseases.* Proceedings of the Third International Symposium, Slovak Academy of Sciences, Bratislava, pp. 62–77.

Regnery RL, Spruil CL, Plikaytis B (1991): Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J. Bacteriol.* **173**, 1576–1589.

Sambrook J, Fritsch EF, Maniatis T (1989): *Molecular Cloning. A Laboratory Manual.* 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor.

Stenos J, Balrd R, Ross B, Bowden S, Dwyer B, Lesus D (1994): Molecular characterization of Rickettsia honei. A newly described member of the rickettsial spotted fever group. In *Program and Abstracts of 11th Sesqui-Annual Meeting of American Society for Rickettsiology and Rickettsial Diseases.* Simons Island, GA, p. 24.

Stoenner HG, Lackman DB, Bell EJ (1962): Factors affecting the growth of rickettsia of the spotted fever group in fertile hen's eggs. *J. Infect. Dis.* **110**, 121–128.

Walker DH (1989): Rickettsioses of the spotted fever group around the world. *J. Dermatol.* **16**, 169.

Weisburg WB, Dobson ME, Samuel JE, Dasch GA, Mallavia LP, Baca O, Mandelco L, Sechrest JE, Weiss E, Woese CR (1989): Phylogenetic diversity of the rickettsiae. *J. Bacteriol.* **171**, 4301–4306.

Weiss E, Moulder JM (1984): Order I. Rickettsiales, Gieszczkiewicz 1939, 25^{AL}. In Kreig NR, Holt JG (Eds): *Bergey's Manual of Systemic Bacteriology.* The Williams and Wilkins Co., The Williams & Wilkins, Baltimore, pp. 687–701.

Webb L, Carl M, Malloy DC, Dash GA, Azad AF (1990): Detection of murine typhus infection in fleas by using the polymerase chain reaction. *J. Clin. Microbiol.* **28**, 530–534.

Yan YS, Uchiyama T, Uchida T (1994): Nucleotide sequence of polymerase chain reaction product amplified from Rickettsia japonica DNA using Rickettsia rickettsii 190 kDa surface antigen gene primers. *Microbiol. Immunol.* **38**, 865–869.

Yu XJ, Jin Y, Fan MY, Xu GM, Liu QH, Raoult D (1993): Genotypic and antigenic identification of two new strains of spotted fever group rickettsiae isolated from China. *J. Clin. Microbiol.* **31**, 83–88.

Zhang JZ, Fan MY, Bi DZ (1995): Detection of spotted fever group rickettsiae from ticks, tick ova, larva, tick faeces and rodent organs by PCR technique in China. *Acta Virol.* **39**, 263–267.

Zhang JZ, Fan MY, Bi DZ (1996): Isolation and identification of new species of spotted fever group rickettsiae. *Chinese J. Zoonoses* **12**, 11–19.