Human and Tick Spotted Fever Group Rickettsia Isolates from Israel: a Genotypic Analysis

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The genomes of spotted fever group rickettsiae isolated in different geographical areas of Israel (two from ticks and four from humans, obtained over a span of 20 years) were studied by polymerase chain reaction (PCR) and restriction endonuclease fragment length polymorphism (RFLP) analysis. The human isolates were obtained from patients suffering from rickettsial disease of different degrees of severity. The PCR products obtained with five pairs of oligonucleotide primers (two primer sets derived from the 190-kDa polypeptide gene and three from the 120-kDa polypeptide gene) and cleaved with restriction endonucleases were used to study the Israeli isolates and reference *Rickettsia conorii* isolates. Subtle differences between the PCR-RFLP patterns of Israeli isolates and the two *R. conorii* reference strains (Moroccan and no. 7) were seen when the PCR products derived from the 190-kDa gene-derived primer sets were digested. All of the Israeli isolates were identical by RFLP analysis using all of the primer sets. This study showed that the Israeli spotted fever group isolates (from both ticks and humans) were genetically homogeneous by the criteria used in this study, despite the time and location differences in their original isolation, and different as a group from *R. conorii*.

Rickettsiae are obligate intracellular gram-negative bacteria. On the basis of antigenic characteristics, they are divided into the spotted fever group (SFG), the typhus group, and the scrub typhus group (12). Mediterranean spotted fever is a tick-transmitted rickettsiosis caused by *Rickettsia conorii*. This disease is endemic to southern Europe, Africa, the Middle East, and the Indian subcontinent (11).

Infection with *R. conorii* can be asymptomatic or symptomatic, ranging from mild to severe with involvement of internal organs. Despite treatment, some patients develop fatal complications. *R. conorii* is especially invasive for the endothelial cells of blood capillaries (10), so that clinical manifestations are those of systemic vasculitis resulting from endothelial damage caused by rickettsial growth within the endothelial cells (3).

During the past 20 years, SFG rickettsiae have been isolated from ticks and human patients with various degrees of disease severity. They have been isolated in different regions of Israel, and they have been identified as *R. conorii* by microimmunofluorescence. Considering the long time span, the different isolate sources (humans and ticks), and the varying severity of rickettsial infection (4) in humans, there is a possibility that different *R. conorii* strains exist in Israel. Use of serological techniques for differentiation of rickettsial isolates is complicated by the significant cross-reactivity between the isolates. Comparison of the DNAs of rickettsiae is an alternative to serologic identification. Restriction fragment length polymorphism (RFLP) analysis of relatively small DNA fragments (polymerase chain reaction [PCR] products) is known to be a useful technique for differentiation and identification of rickettsiae (6-8).

In the present study, we used PCR-RFLP techniques to

investigate the possible existence of genomic differences among Israeli SFG isolates from either patients or ticks and between such isolates and reference isolates.

MATERIALS AND METHODS

Rickettsial isolates. The SFG isolates used in this study, dates of isolation, and places of isolation are shown in Table 1. The Israeli isolates came from different geographical locations over a period of 20 years, and the human isolates represent diverse examples of clinical disease severity. They include isolates from patients with clinically mild (R-358 and G-212), clinically severe (A-828), and fatal (S-484) illnesses. Isolates S-484, G-212, T-487, and T-192 were kindly supplied by Robert Goldwasser. Isolates G-358 and P-828 were isolated in the rickettsial laboratory, Virology Unit, Ben-Gurion University. The passage histories of the various isolates used in this study varied considerably.

Rickettsial propagation. BGM cells (an African green monkey kidney line) were grown in 150-cm² flasks (Nunc, Roskilde, Denmark) in antibiotic-free RPMI 1640 medium supplemented with 10% fetal calf serum and 1% glutamine. After 48 h, the cells were infected with rickettsiae at a multiplicity of infection of 1 to 2. After 2 h of adsorption, medium containing 1 µg of cycloheximide per ml was added to the infected cells. The cells were incubated at 35°C with 5% CO₂. At 96 h postinfection, when 90 to 100% of the cells showed a cytopathic effect, glass beads were added to each flask and the cells were detached by gentle shaking and sonicated for 30 s. Debris was pelleted by 10 min of centrifugation at 250 × g. The supernatant was then centrifuged for 10 min at 26,000 × g. Five milliliters of buffer (Tris [pH 7.6], NaCl at 150 mM, EDTA at 2 mM) was added per 150-cm² cell culture flask pellet. Cytoplasmic DNA extracts were made from the resuspended pellet as described previously (7).

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TABLE 1. Rickettsial strains

Rickettsial isolate	Origin	Year of isolation	Location or strain designation	Passage history ^a
A-828	Human	1988	Southern Israel	TC ^b
G-212	Human	1971	Israel coastal plain	GP-E-TC ^c
R-359	Human	1990	Southern Israel	TC ^b
S-484	Human	1972	Israel coastal plain	GP-E-TC ^c
T-487	Hedgehog tick	1970	Israel coastal plain	E-GP-TC ^c
T-162	Tick	1971	Israel coastal plain	E-GP-TC ^c
Moroccan	Tick	?	ATCC VR-141	?
No. 7	Tick	?	ATCC VR-613	?

^a E, embryonate eggs; GP, guinea pig; TC, tissue culture.

^b Four to eight passages in tissue culture.

^c Over 20 passages in tissue culture.

PCR amplification. PCR amplification was accomplished in 100- μ l volumes by using the protocols supplied with the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.). Typically, 1 μ l of undiluted cytoplasmic extract DNA was used as the PCR template. Sets of PCR reactions always included a control reaction containing no template DNA and another control containing material extracted from uninfected Vero cells. DNA amplification was done in a Perkin-Elmer Cetus DNA Thermal Cycler by using 35 cycles of denaturation (20 s at 95°C), annealing (30 s at 48°C), and extension (2 min at 60°C).

DNA digestion and electrophoresis. PCR amplification of DNA was verified by rapid agarose electrophoresis of a small amount of the PCR product. Restriction endonuclease digestion (see Table 3) was done with 20 μ l of the PCR reaction mixture by following standard techniques (9). All restriction endonucleases selected had at least two restriction sites within the PCR-amplified DNAs and were obtained from New England BioLabs, Beverly, Mass. After addition of a dye-Ficoll loading mixture (9), the digested reactions were loaded on 1.5-mm-thick, 8% polyacrylamide (Bio-Rad Laboratories, Richmond, Calif.) vertical gels made by standard procedures (9). Gels were run at 80 V for 4 h in simple vertical electrophoresis chambers (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.). The gels were then stained with ethidium bromide and photographed under a UV light source (365 nm; Spectronic Corp., Westbury, N.Y.). Diagnostic PCR-RFLP patterns for all isolates were obtained for at least three different digests of five different amplified products (see Table 3).

Oligonucleotide primers. The oligonucleotide primers used are shown in Table 2. Oligonucleotide primers for PCR amplification of regions of the 190-kDa SFG antigen gene were derived from the *R. rickettsii* (R strain) sequence.

Primer pairs are referred to by the initials of the genus and species from which the nucleotide sequence was derived, combined with a simple alphanumeric identifier of the gene amplified, and followed by the position of the first nucleotide of the positive strand of the primer (using conventional 5'-3' presentation of sequence data) and then the position of the last nucleotide of the negative strand of the corresponding primer from the complementary strand of DNA. The specificities of these primers have been reported previously (7).

Nucleotide primers were produced by the Division of Viral and Rickettsial Diseases, Biotechnology Resource Facility, Centers for Disease Control.

RESULTS

Genomic differences between Israeli SFG rickettsia isolates (Table 1) were studied by RFLP analysis of DNA sequences from specific genes (for the 190- and 120-kDa polypeptides) amplified by PCR. The products amplified by using five pairs of oligonucleotide primers (two derived from two areas of the 190-kDa polypeptide gene and three derived from the 120-kDa polypeptide gene) (Table 2) and cleaved by restriction with the enzymes listed in Table 3 were used to differentiate among the Israeli SFG isolates and between these and the reference *R. conorii* isolates (Morocco and no. 7). PCR products produced by using all of the sets of primers and digested with all of the restriction endonucleases (Table 3) showed that all of the Israeli SFG isolates were identical (Fig. 1 to 3). Figures 1 and 2 show the results for one human isolate (A-828) and one tick isolate (T-487), as well as two reference *R. conorii* strains (Morocco and no. 7). PCR products obtained with primer set I digested with *Pst*I, *Rsa*I, *Alu*I, and *Fnu*4HI, primer set II digested by *Rsa*I, *Alu*I, and *Dde*I, and primer set III digested by *Fnu*4HI showed that the Israeli isolates differ from the two *R. conorii* reference strains (Fig. 1 and 2). PCR-RFLP analysis using primer sets

TABLE 2. Oligonucleotide primers

Set no. and primer	Size (kDa) of antigen coded for by gene	Nucleotide sequence (5'-3')	Product size (bp)
I Rr190.94p Rr190.626n	190	ATGGCGAATATTTCTCCAAAA AGTGCAGCATTGCTCCCCCT	532
II Rr190.4441p Rr190.5663n	190	CGATAGCCGGAGGCAAGACGT TGCAAGCGGTCCACCCGGTGC	1,222
III Rr120.150p Rr120.800n	120	GGCAATTAATATCGCTGACGG GCATCTGCACTAGCACTTTT	650
IV Rr120.1585p Rr120.2200n	120	GGGGTTAATGTTGATACTG CACCTAAACCAACGACGC	615
V Rr120.3710p Rr120.4120n	120	GCTATCGGTATCACTAAAACTG CGGGAGCGATTAGAAGTTTACACGG	410

TABLE 3. Primer sets and restriction enzymes used and RFLP differences between Israeli and reference strains

Enzyme	Primer set ^a :				
	I	II	III	IV	V
<i>Pst</i> I	Different				
<i>Rsa</i> I	Different	Different	Used	Used	Used
<i>Alu</i> I	Different	Different	Used	Used	
<i>Dde</i> I		Different			Used
<i>Msp</i> I		Used		Used	Used
<i>Fnu</i> 4HI	Different		Different		
<i>Taq</i> I			Used		

^a Primer sets are defined in Table 2.

derived from the 120-kDa polypeptide gene cleaved by the enzymes shown in Table 3 showed no divergence either among Israeli SFG isolates or between Israeli SFG isolates and *R. conorii* reference strains (Fig. 3), except primer set III digested by *Fnu*4HI.

DISCUSSION

RFLP analysis of relatively short segments of PCR-amplified DNA minimizes the need for structurally intact genome DNA in genotypic analysis. Small differences between DNA

FIG. 2. Ethidium bromide-stained polyacrylamide gel electrophoretograms of *Dde*I (A) and *Alu*I (B) restriction endonuclease-digested, PCR-amplified SFG rickettsial DNA derived from the 190-kDa antigen gene, analyzed by using primers Rr190.4441p and Rr190.5663n. SFG rickettsiae are represented by *R. conorii* no. 7 (#7), Morocco (M), Israeli human isolate (IH), and Israel tick typhus (IT). ϕ X174 replicative-form DNA cleaved with *Hinf*I was used as a size standard adjacent to each rickettsial sample.

FIG. 1. Ethidium bromide-stained polyacrylamide gel electrophoretograms of *Alu*I (A), *Pst*I (B), and *Fnu*4HI (C) restriction endonuclease-digested, PCR-amplified rickettsial DNA derived from the 190-kDa antigen gene and analyzed by using primers Rr190.94p and Rr190.626n. SFG rickettsiae are represented by *R. conorii* no. 7 (#7), Morocco (M), Israeli human isolate (IH), and Israel tick typhus (IT). ϕ X174 replicative-form DNA cleaved with *Hinf*I was used as a size standard adjacent to each rickettsial sample.

fragments are readily resolved by polyacrylamide gel electrophoresis. PCR amplification utilizing oligonucleotide primers which are well conserved among SFG rickettsiae provides a convenient source of isolate-specific DNA for RFLP analysis (7). PCR-RFLP analysis allows identification and partial characterization of various SFG rickettsia isolates from Israel.

All of the Israeli SFG rickettsial isolates in the present study were identical by PCR-RFLP analysis. These isolates originated from different hosts in various regions in Israel over a period of 20 years. The human isolates were derived from patients who had various clinical outcomes of disease ranging from mild to fatal complications. This study suggests that all Israeli SFG rickettsial isolates belong to a single genotype. This genotype has apparently remained stable during transmission from ticks to humans and during multiple passages in experimentally infected animals, embryonated eggs, and tissue culture. Nonetheless, all of the Israel isolates of *R. conorii* can be distinguished from the no. 7 and Moroccan *R. conorii* reference isolates by subtle differences

FIG. 3. Ethidium bromide-stained polyacrylamide gel electrophoretograms of *Dde*I restriction endonuclease-digested, PCR-amplified SFG rickettsial DNA derived from the 120-kDa antigen gene, analyzed by using primers Rr120.3710p. and Rr120.4120n. SFG rickettsiae are represented by *R. conorii* no. 7 (#7), Morocco (M), Israeli human isolate (IH), and Israel tick typhus (IT). ϕ X174 replicative-form DNA cleaved with *Hinf*I was used as a size standard adjacent to each rickettsial sample.

in PCR-RFLP patterns, as previously reported for a single SFG rickettsial isolate from Israel (7). These results may corroborate those previously demonstrated by immunofluorescence (4) and/or gel electrophoresis (2). Furthermore, they underscore the discriminating power and ease of PCR-RFLP analysis. Several human isolates of *R. conorii* isolated in Spain and France were identical to one another by PCR-RFLP analysis and also identical to the no. 7 reference strain of *R. conorii* (2a). Therefore, in contrast to the apparent genetic homogeneity of human isolates of *R. rickettsii* from various regions of the western hemisphere (7), our PCR-RFLP analysis suggests that the subtle differences previously noted among *R. conorii* genotypes from human isolates may be consistent with distinct geographic distributions.

The genotypic homogeneity of the Israeli *R. conorii* isolates, coupled with the finding that various human *R. conorii* isolates from France and Spain represent a separate but homogeneous genotype, suggests that *R. conorii* strains in the western and eastern Mediterranean regions have been subjected to a level of genetic isolation. The factors that may influence this genetic isolation are unknown but perhaps relate to subtle differences in the vectors and/or vertebrate hosts which are peculiar to the various regions. Long-term geographic isolation may also be a factor in genotypic divergence; however, it seems likely that multiple opportunities for exchange of genotypes have occurred, especially during periods of human migration and transport of vertebrate hosts and tick vectors. Genotypic analysis of additional *R. conorii* isolates from throughout the range of this widespread rickettsial species should prove interesting for delineation of the distribution of various genotypes and thereby testing of the hypothesis of geographical or ecological differences in *R. conorii* genotypes. This, in turn, may provide important clues for a better understanding of the evolution of SFG rickettsiae.

The 190-kDa antigen found among various SFG rickettsiae (1) is thought to be exposed on the surface of rickettsiae (5) and therefore may be subjected to increased evolutionary pressure related to host defense mechanisms, as well as host cell receptors. The variability in the nucleotide sequences from this gene, as evidenced by PCR-RFLP analysis, from various SFG rickettsial genotypes and species (7) may be a reflection of these evolutionary pressures. In contrast, we have found that the PCR-RFLP patterns derived from the 120-kDa antigen gene were better conserved than those derived from the 190-kDa antigen gene, suggesting either that the 120-kDa gene product is exposed to fewer evolutionary pressures than the 190-kDa antigen or, alternatively, that genetic variation within this gene cannot be tolerated to preserve a critical phenotypic function. Whatever the underlying reason for relative genetic conservation, our results show that the 190-kDa antigen gene is a more useful substrate for genotypic analysis of SFG rickettsiae (by PCR-RFLP analysis) than is the 120-kDa antigen gene.

Pathogen virulence factors and variable host immunity are two probable sources of variability in disease outcome. Although the PCR-RFLP analysis is not designed to show all

possible genetic differences in pathogen genetics, the method can be used as a valuable method for sampling genetic variability. Our results suggest that the Israeli SFG isolates are homogeneous by the criteria used in this analysis, which lends support to the suggestion that differential outcomes of *R. conorii* infections are due to variable host responses.

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REFERENCES

1. Anderson, B. E., G. A. McDonald, D. C. Jones, and R. L. Regnery. 1990. A protective protein antigen of *Rickettsia rickettsii* has tandemly repeated, near-identical sequences. *Infect. Immun.* **58**:2760-2769.
2. Cwikel, B. J., J. Igbarieh, and I. Sarov. 1990. Antigenic peptides of Israeli spotted fever isolates compared with other spotted fever group rickettsiae. *Ann. N.Y. Acad. Sci.* **590**:381-388.
- 2a. Drancourt, M., and R. L. Regnery. Personal communication.
3. Gear, J. H. S. 1988. Other spotted fever group rickettsioses: clinical signs, symptoms, and pathophysiology, p. 101-138. *In* D. H. L. Walker (ed.), *Biology of rickettsial diseases*, vol. 1. CRC Press, Inc., Boca Raton, Fla.
4. Goldwasser, R. A., Y. Steiman, W. Klingberg, T. A. Swartz, and M. A. Klingberg. 1974. The isolation of strains of rickettsiae of the spotted fever group in Israel and their differentiation from other members of the group by immunofluorescence. *Scand. J. Infect. Dis.* **6**:53-62.
5. McDonald, G. A., R. L. Anaker, and Kareen Garjian. 1987. Cloned gene of *Rickettsia rickettsii* surface antigen: candidate vaccine for Rocky Mountain spotted fever. *Science* **235**:83-85.
6. Regnery, R. L., and C. L. Spruill. 1984. Extent of genetic heterogeneity among human isolates of *Rickettsia prowazekii* as determined by restriction endonuclease analysis of DNA, p. 297-300. *In* D. Schlessinger and L. Levine (ed.), *Microbiology—1984*. American Society for Microbiology, Washington, D.C.
7. Regnery, R. L., C. L. Spruill, and B. D. Plikaytis. 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J. Bacteriol.* **173**:1576-1589.
8. Regnery, R. L., T. Tzianabos, J. J. Esposito, and J. E. McDade. 1983. Strain differentiation of epidemic typhus rickettsiae (*Rickettsia prowazekii*) by DNA restriction endonuclease analysis. *Curr. Microbiol.* **8**:355-358.
9. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. Walker, D. H., and W. D. Mattern. 1980. Rickettsial vasculitis. *Am. Heart J.* **100**:896-906.
11. Wang, J. G., and D. H. Walker. 1987. Identification of spotted fever group rickettsiae from human and tick sources in the People's Republic of China. *J. Infect. Dis.* **156**:665-669.
12. Weiss, E., and J. W. Moulder. 1984. Genus I. *Rickettsia*, p. 688-698. *In* N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.