POLYMERASE CHAIN REACTION–BASED DIAGNOSIS OF MEDITERRANEAN SPOTTED FEVER IN SERUM AND TISSUE SAMPLES

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Abstract. A nested polymerase chain reaction (PCR) assay has been developed and used in the diagnosis of fatal and benign cases of Mediterranean spotted fever (MSF). The test was based on specific primers derived from a Rickettsia conorii 17-kD protein gene. A positive signal was obtained from spotted fever group (SFG) and typhus group (TG) rickettsiae. Discrimination between SFG and TG rickettsiae was based on a restriction fragment length polymorphism test. Other gram-negative bacterial species tested did not generate a signal, attesting for the specificity of the assay. The SFG-specific DNA fragment was detected in four of 29 acute-phase sera from serologically confirmed patients with MSF, while acute-phase sera from 25 patients without MSF were PCR negative. Acute-phase sera samples (five of five) and tissue autopsies (six of seven) from fatal suspected cases of MSF were PCR positive. The results demonstrate that sera and tissue samples are suitable specimens for the nested PCR tests, especially in fatal cases.

INTRODUCTION

Mediterranean spotted fever (MSF), an endemic disease in Israel,1 is caused by Rickettsia conorii, which is a strict intracellular, slow-growing, gram-negative bacterium. The disease is usually characterized by the onset of high fever, myalgia, arthralgia, and a typical rash. However, in some cases the patients present with multi-organ failure and die within a few days. In most cases, diagnosis of MSF is based on serology. Since antibodies can be detected in most cases 7–10 days after the onset of the disease, diagnosis in the acute phase must rely on the detection of rickettsiae in the clinical specimens.

Detection of rickettsial DNA is based on amplification of specific sequences of the genes encoding the 16S rRNA, the 17-kD protein, citrate synthase (gltA), and the outer membrane proteins OmpA and OmpB.2 The gene encoding the 17-kD protein shows high homology among the spotted fever group (SFG) and typhus group (TG) rickettsiae, indicating that its sequence represents a suitable target for diagnosis of Rickettsia species.3 We have previously reported the use of primers derived from the R. rickettsii 17-kD gene to detect rickettsial DNA in a blood sample from a fatal, serologically unconfirmed case.4 Although 17-kD protein gene sequences from R. rickettsii and R. conorii show high homology, a few mismatches within the primer sequences exist; thus, the efficiency of detection of R. conorii may be limited. In this report, we demonstrate the use of new sets of primers, which are highly specific for the R. conorii 17-kD protein gene (RCANT17K), for the detection of R. conorii in various clinical specimens.

MATERIAL AND METHODS

Bacteria. The following bacterial species were used in this work: Rickettsia conorii strains FB Moroccan, ISF–Israeli 467, Kenya tick typhus (KTt), and South African tick typhus (SATT); R. australis (NQTT New Queensland tick typhus); R. rickettsii Sheila-Smith strain; R. akari; R. typhi Wilmington strain; R. prowazekii Breinl strain; Coxiella burnetii Nine-Mile strain; Ehrlichia canis 611 Israeli strain; E. chaffeensis Arkansas strain; human granulocytic ehrlichiosis (HGE) agent NCH strain; Salmonella sp. (local isolate); and Escherichia coli.

Rickettsia spp. were grown in yolk sacs of embryonated hen eggs, as described previously.5 Quantitative estimation of R. conorii and R. typhi was obtained by counting immunofluorescence-stained rickettsiae.

Extraction of DNA. Bacterial suspensions were centrifuged in a minifuge, and the pellet was resuspended in buffer (4 mM Tris pH 8.0, 0.25 mM EDTA, 10% Triton X-100) and incubated for 30 minutes at 100°C. The DNA was extracted from sera (∼0.2 mL), skin biopsy samples, and paraffin-embedded tissues samples (25 mg) using the QIAmp kit (Qiagen GmbH, Hilden, Germany). The final volume of the extracted DNA was 50 µL.

Primer selection. Two sets of primers that amplified the 17-kD protein gene sequences of R. conorii (RCANT17K) were synthesized. Selection of the primers was based on the “Primer 0.5” program6 to obtain optimal Tm and GC content, and to avoid hairpin loop structures. The selected sequences were analyzed by the “BLAST” program7 to identify primers for detecting the various SFG Rickettsia species and to minimize the similarity of the primers to other bacterial or human sequences.

PCR conditions. The PCR mixture (50 µL) contained 10 mM Tris pH 9.0, 50 mM KCl, 2.5 mM MgCl2, 0.1% Triton X-100, 200 µM dNTPs, 25 pmoles of each primer, and two units of Taq DNA polymerase (Promega, Madison, WI). Samples (5 µL) were amplified for 30 repeated cycles of denaturation at 94°C, annealing at 55°C, and extension at 72°C, each for 20 seconds, followed by an additional extension at 72°C for two minutes. The PCR products were analyzed by gel electrophoresis (2% agarose). Confirmation of fragment size was based on DNA molecular weight marker XIII (Boehringer, Mannheim, Germany). Restriction enzyme digestion (Bfa I; New England Biolabs, Beverly, MA) was performed according to the manufacturer for all positive nested PCR samples. To avoid cross-contamination we have used strict regulations recommended for PCR diagnostic laboratories.8

Clinical samples. Samples were submitted to the Israeli National Reference Center for Rickettsioses along with patient histories. Patients who did not convert serologically to SFG rickettsiae were considered as patients without MSF.

Immunofluorescence assay (IFA). The IgG and IgM titers were determined by IFA as described previously.5
RESULTS

Establishment of a nested PCR test for detection of *R. conorii*. To detect the gene (539 basepairs) encoding the 17-kD protein, we designed two sets of primers designated rickP3/P2 and rickP5/P4. They encompassed the following sequences: rickP3 - GGAACACTTCTTGGCGGTG, rickP2 - CATTGTCCGTCAGGTTGCCG, rickP5 - GCATTTCTGTTATCAATTCGG, and rickP4 - AACCCTTAATTGCGGTATCCCG. A homology search (BLAST) of the primers rickP3 and rickP4 indicated full homology with SFG rickettsiae such as *R. conorii*, *R. rickettsii*, *R. parkeri*, *R. rickettphi*, *R. australis*, and *R. akari* (score = 110), while they showed lower similarity (score < 80) to TG rickettsiae, *R. typhi*, and *R. prowazekii*. The two other primers, rickP5 and rickP2, exhibited high homology for both SFG and TG rickettsial groups (scores of 115 for primer rickP5 and 100 for primer rickP2). The primers rickP3 and rickP2 amplified a 371-basepair (bp) DNA fragment of the 17-kD protein gene of *R. conorii* (flanking positions 153-523). The primers rickP5 and rickP4 amplified a nested 214-bp DNA fragment (flanking positions 180-393). The sensitivity of the PCR tests was evaluated with *R. conorii* and *R. typhi* suspensions with known concentrations of rickettsial particles. The sensitivity based on rickP3/rickP2 primers was 100 and 10,000 particles per assay for *R. conorii* and *R. typhi*, respectively. The use of a nested PCR test with the rickP5/rickP4 primers increased the detection sensitivity for both *Rickettsia* species to < 10 rickettsial particles per assay (Figure 1A). Other gram-negative bacteria such as *Coxiella burnetii*, *Ehrlichia canis*, *E. chaffeensis*, *HGE* agent, *Escherichia coli*, and *Salmonella* sp. did not produce any specific DNA fragment with our PCR system, attesting to its high specificity for *Rickettsia* species.

The universality of primers in detecting rickettsial strains was confirmed with DNA extracted from various rickettsiae: *R. conorii* isolates (from Morocco, Israel, Kenya, and South Africa), *R. australis*, *R. rickettsii*, *R. akari*, *R. typhi*, and *R. prowazekii*. Analysis of the nested PCR products revealed the expected DNA fragment with all these species. Despite the fact that the oligonucleotide primers are not fully matched to the 17-kD protein gene of the TG rickettsiae, the detection of this specific DNA fragment conceivably stems from the high sensitivity of the nested PCR test.

To distinguish between SFG and TG rickettsiae, we used a restriction fragment length polymorphism (RFLP) test based on digestion of the 214-bp DNA PCR product with the restriction enzyme *Bfa* I. The SFG rickettsiae nested PCR amplicon contains one *Bfa* I site (position 343), resulting in the generation of two fragments (164 bp and 50 bp), while the TG rickettsiae amplicon contains two sites (positions 302 and 339), thereby resulting in the generation of three fragments (127 bp, 50bp, and 37bp; Figure 1B).

**Analysis of clinical specimens by the PCR.** The nested PCR test was applied to detect the presence of rickettsial DNA in various specimens: acute-phase sera, skin biopsy samples, and tissues samples collected at autopsies. A typical test of an acute-phase serum of a serologically confirmed patient with MSF is shown in Figure 1C. In the first PCR, no positive signal could be detected, while the nested PCR test resulted in a significant positive signal (214-bp DNA fragment).

The DNA was extracted from various clinical specimens and subjected to the PCR tests. All acute-phase sera tested from patients without MSF (n = 25) were negative in the nested PCR. Fourteen percent of the serologically confirmed cases with MSF (4 of 29, patients 1–4) and all the available sera from fatal cases (n = 5, patients 6–10) were positive in the nested PCR (Table 1). In addition, the nested PCR test was used to detect the presence of rickettsial DNA in tissue samples (skin, spleen, kidney, and testis). Most of the tissue specimens (except testsis) were positive in the nested PCR (patients 5, 6, 11, and 12). All positive nested PCR samples were found to be of SFG origin by the RFLP test. In the first PCR, three of 16 samples were positive for MSF: an acute-phase serum from a fatal case, a skin biopsy sample from a patient with benign MSF, and a spleen autopsy sample. The positive signals from the other 13 samples were obtained only by performing the nested PCR test. In eight cases, the positive results provided by the nested PCR tests were supported by other assays or by circumstantial evidence (Table 1). The positive PCR results of acute-phase sera from four patients (1–4) showed a correlation with serum conversion that appeared during the convalescence period. Rickettsial DNA was detected in a skin biopsy sample taken from the rash site of another serologically confirmed case (patient 5) with MSF. In two fatal cases (patients 6 and 7), SFG rickettsiae were observed in tissues samples by immunostaining techniques. In two other fatalities (patients 6 and 12), morbidity of another family member was diagnosed as MSF by serology.

DISCUSSION

Laboratory diagnosis of MSF is often determined by serology. However, during the first few days of the disease, when antibodies are not detectable, diagnosis should be based on the detection of the rickettsiae in clinical samples. Rickettsiae can be detected using procedures such as isolation, immunohistochemical staining, and the PCR.

The PCR assay described in this study is based on the amplification of an *R. conorii* 17-kD common antigen gene sequence. Since this gene is highly conserved among *Rickettsia* species, the selected primers are able to detect both SFG and TG rickettsiae. Positive results were obtained with several SFG and TG members. The discrimination between the two groups is based on an RFLP test that exploits *Bfa* I digestion of the 214-bp amplicon. Since murine typhus is endemic in Israel, the use of the RFLP test is required to avoid misinterpretation of the results.

The PCR test enables the detection of 100 rickettsiae in a relatively short time. The sensitivity of the nested PCR test is enhanced by two orders of magnitude, which increases the probability of detecting rickettsial DNA in the clinical samples. Thus, although only three of the 16 positive results were seen in the first PCR, the other 13 were shown to be positive only by using the nested PCR test.

Since rickettsiae are intracellular, whole blood or buffy coat are considered preferable samples for the PCR tests. However, most of the specimens provided by medical centers were serum samples. The nested PCR test detected SFG rickettsial DNA in four (14%) of 29 acute-phase sera of patients with serologically confirmed MSF, and in all available sera (five of five) of suspected fatal cases of MSF. La Scola and Raoult reported negative PCR results in 41 sera samples from...
patients with MSF. Our positive results may be due to the higher sensitivity of the nested PCR test.

The PCR test was very useful in the diagnosis of MSF in serologically unconfirmed fatal cases. All available acute-phase sera and most of the tissue samples were positive in the nested PCR. The results obtained with samples originating from fatal cases indicate that the sera contained at least 50 rickettsial DNA copies per milliliter and the tissues contained at least 400 copies per gram. Similar findings were reported by Tzianabos and Paddock and others for the diagnosis of Rocky Mountain spotted fever, which is caused by R. rickettsii, in blood clots and tissue specimens of fatal cases.

In conclusion, we have developed a sensitive and specific PCR test based on sequences of the 17-kD protein gene of R. conorii that can be used in the diagnosis of MSF. The test can be used to detect the presence of SFG rickettsiae in sera and
skin biopsy samples obtained during the acute phase of the disease and for the diagnosis of MSF in fatal cases.

Acknowledgments: We thank Levana Motola and Carmela Strenger for excellent technical assistance.

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REFERENCES


### TABLE 1

PCR results from clinical specimens of suspected patients with MSF*  

<table>
<thead>
<tr>
<th>Course of the disease</th>
<th>Patient no.</th>
<th>Acute-phase serum</th>
<th>Tissues†</th>
<th>Supportive evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>1, 2, 3</td>
<td>Positive</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Positive</td>
<td>–</td>
<td>SFG rickettsiae were isolated from a tick picked from the patient’s dog.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Negative</td>
<td>Skin biopsy positive</td>
<td>–</td>
</tr>
<tr>
<td>Fatal</td>
<td>6</td>
<td>Positive</td>
<td>Spleen, kidney positive</td>
<td>SFG rickettsiae were identified by IFA in sections from the spleen; another family member was diagnosed as a patient with MSF.</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Positive</td>
<td>NA</td>
<td>SFG rickettsiae were identified by immunohistochemistry (IHC) in kidney and CNS.9</td>
</tr>
<tr>
<td></td>
<td>8, 9, 10</td>
<td>Positive</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>NA</td>
<td>Spleen, skin positive</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>NA</td>
<td>Spleen, lung positive Testis negative</td>
<td>A tick picked from the patient’s dog was PCR positive; another family member was diagnosed as a patient with MSF.</td>
</tr>
</tbody>
</table>

NA—non available.  
†Tissue samples from patients 6 and 11 were formalin-fixed, and paraffin-embedded.  
* PCR = polymerase chain reaction; MSF = Mediterranean spotted fever; SFG = spotted fever group; IFA = immunofluorescent assay; IHC = immunohistochemical analysis; CNS = central nervous system.