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CASE REPORT

First Isolation of *Rickettsia conorii* from Human Blood in Croatia

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Aim. To detect and isolate rickettsial strains from blood samples of patients with presumptive diagnosis of Mediterranean spotted fever (MSF) in the coastal region of south Croatia, and to compare the results with routine serology.

Methods. A "suicide" polymerase chain reaction (PCR), and a shell vial culture were done on samples of ethylenediamine tetra-acetic acid (EDTA) and citrate-anticoagulated blood samples. Indirect immunofluorescence was performed on sera collected from 17 patients clinically diagnosed with MSF during summer in three consecutive years, from 1998 to 2000.

Results. The primers used in PCR amplified the expected part of the rickettsia genomic DNA and *Rickettsia conorii* grew from the shell vial-cultured blood of a single patient. In 13 patients, the diagnosis was confirmed serologically by paired sera, whereas in 4 patients the diagnosis remained presumptive, since no paired sera were available. Analyzing sequences of the ompA and citrate synthase gene, respectively, derived from the shell vial isolate, a 100% similarity with *Rickettsia conorii*, strain Seven (Malish), was found.

Conclusion. To the best of our knowledge, this is the first isolation of *Rickettsia conorii* from a human sample in Croatia, and the first proof of a causative agent of MSF in the country. Beside PCR-based methods and isolation, correct diagnosis of MSF could be still routinely reached by serology.

Key words: *Boutonneuse fever; polymerase chain reaction; Rickettsia conorii; rickettsia infections; sequence analysis; serology*

Spotted fever group rickettsiae are obligate intracellular bacteria, mostly tick-transmitted, and capable of infecting vertebrates, including humans (1). *Rickettsia conorii* is the predominant etiologic agent of Mediterranean spotted fever, also called boutonneuse fever or Marseille fever, transmitted in the Mediterranean area by *Rhipicephalus sanguineus*, the dog brown tick (1).

Geographically, *Rickettsia conorii* is widely distributed and can be found in southern Europe, Africa, the Middle East, and India (2). Phylogenetic studies of strains from different regions or vectors clearly demonstrate their diversity, which is reflected in antigenic variations (3,4). Those studies have shown that *Rickettsia conorii* strain Seven (Malish), the proposed type strain (5), clusters together with Israeli tick typhus and Astrakhan fever rickettsial strains. These strains, although isolated from widely separated parts of the world, form genetically homogenous *Rickettsia conorii* complex (6). Increasing number of new rickettsiae appears in different regions of the world, partly due to the advanced methods of isolation and identification of these bacteria; atypical cases and many new clinical syndromes have been described (1).

Soon after the first published reports on Mediterranean spotted fever in Africa and Europe in the first third of 20th century (7-9), the clinical presentation comparable to that fever was also reported in Split (10), a Croatian city located on the eastern coast of the Adriatic Sea. Several seroepidemiological studies performed in Croatia about 50 years later revealed the presence of this exanthematic disease in the whole coastal region of the country (11-17).

According to the data published in these reports, the prevalence of antibodies to spotted fever group rickettsiae in the Croatian coast ranged from 15% (in the most southern parts) to 44% (in central and northern parts) (16,17). Furthermore, investigations on ticks collected along the Croatian coast showed not only the presence of *R. conorii* in dog ticks, namely *R. sanguineus*, but also the existence of different tick genera in this region, implying the possibility of different rickettsial species to be transmitted to humans causing spotted fever disease (16,18,19).

Until now, no rickettsial isolate of human origin was obtained from patients with Mediterranean spotted fever in Croatia. In most cases, the diagnosis of Mediterranean spotted fever was based on clinical findings and confirmatory results of serological tests. However, since the immunological response is delayed and strong serological cross-reactions between species do not allow complete strain differentiation (1,20), direct and rapid evidence of identity of rickettsial pathogen is required. It should be based on the detection of the rickettsiae in clinical samples.

In this study, beside routine serology, we performed polymerase chain reaction (PCR) detection, isolation, and identification of rickettsiae from the blood of patients with clinical pictures comparable to Mediterranean spotted fever in the region of south Croatia.

Patients and Methods

During the 1998-2000 period, 17 patients, aged 4-72 years and with clinical and epidemiological findings suggestive for Mediterranean spotted fever, were admitted to the Department of Infectious Diseases of the University Hospital Split, Split, Croatia. Regarding the epidemiologic criteria as a part of the scoring system for diagnosis of Mediterranean spotted fever (21), they all lived in endemic Adriatic coastal area, the onset of their disease was between May and September, and 11 out of 17 recalled contact with dog tick(s). Clinically, all of them had two out of three major findings – fever >39 °C and maculopapular or purpuric eruption. In addition, eight of them had eschar (*tache noire*) (21).

On their first and third hospital day, 5 mL of the whole blood with ethylenediamine tetra-acetic acid (EDTA), 5 mL of blood with citrate, and 5 mL of blood without additives (only on the first day and during the control visit) were collected and sent to the Department of Clinical Microbiology. The EDTA- and citrate-blood samples were immediately frozen and kept at -80 °C until shipment on dry ice to France, where PCR and culture were performed.

For serological testing, sera were separated from blood without additives and kept at -20 °C, until testing.

Detailed Report of a Patient with Positive PCR/Culture

In August 1998, a 67-year-old man with acute febrile illness was admitted to the hospital. He was a worker in an orchard. His neighbor had a dog, but he claimed no direct contact with dogs or ticks, and did not remember being bitten by a tick. The illness began seven days prior to hospital admission, with a fever of 39-40 °C, chills, sweating, headache, and joint and muscle pains. On the third day of illness a non-itching rash appeared on his body, arms, and legs. On admission, he was febrile, with a generalized maculopapular rash, which also involved palms and soles. He had a 0.5-1 cm black crust (*tache noire*) above the right iliac crest surrounded by an erythematous halo. Laboratory test results were as follows: erythrocyte sedimentation rate, 45 mm/h; leukocyte and platelet counts, 5.1 x 10^9/L and 64 x 10^9/L, respectively; blood urea nitrogen and creatinine, 14.9 mmol/L and 156 mmol/L, respectively; alanine aminotransferase, 67 U/L; aspartate aminotransferase, 176 U/L; lactate dehydrogenase, 921 U/L; and creatinine phosphokinase, 679 U/L. A presumptive diagnosis of Mediterranean spotted fever was made and treatment with 100 mg doxycycline orally per day was immediately initiated and continued for 10 days. On day four of the treatment, the patient became afebrile and remained in good clinical condition.

Shell Vial Culture with Detection of Rickettsiae and Propagation of the Strain

Blood samples with citrate collected on the first and on the third day of hospital stay were used for culture by inoculating three shell vials seeded with human embryonic lung (HEL) fibroblasts, as described elsewhere (22,23).

On the sixth day of culture, a small portion of the cell monolayer on the cover slip was scraped and put on a slide for Gimenez staining (24). The cover slip was also stained by indirect immunofluorescence, as described previously in details (22,23). When immunofluorescence was positive, the supernatant and the cells on the cover slip from the parallel shell vial were further propagated to obtain an isolate for definitive identification by PCR and sequencing (22,23).

PCR of the Blood and Culture Sample

Genomic DNA was extracted from 200 µL of EDTA-blood by using Qiagen columns (QIA amp® DNA MINI Kit, Qiagen, Courtaboeuf, France), according to the manufacturer's recommendations. A "suicide" PCR was performed (25) with primers never before applied in the laboratory, targeting the ompA gene (GenBank accession number U43806), namely SLO 1F and SUI 1R for the first PCR, and SLO 2F and SUI 2R for the nested PCR amplification (Table 1). The primers were purchased from Eurobio (Paris, France). To avoid the risk of contamination, no positive control was used.

Table 1. Oligonucleotide primers used for "suicide" polymerase chain reaction (PCR) amplification of DNA extracted from blood samples (ompA gene)

Primer	Nucleotide sequence (5' - 3')	*ompA* position	Fragment size (bp)
SLO 1F	CAC CAC CTC AAC CGC AG	136-152	464
SUI 1R	TTT GCC GGG GCT GCA GAT	633-616	
SLO 2F	GGG GCA CTC GGT GTT GC	173-189*	386
SUI 2R	TCC TAA ACC TGT ATA ATT ATC G	574-553*	

*Primers used for sequencing.

The success of the amplification was confirmed by resolution of the products by electrophoresis on 1% agarose gel (Sigma Chemical Co., St. Louis, MO, USA) in 1xTris borate EDTA buffer for 1 h. The sizes of the PCR-amplified products were determined by comparison with a molecular weight standard (marker VI; Boehringer, Manheim, Germany) under ultraviolet light following ethidium bromide staining.

For PCR from the culture sample, extraction of genomic DNA was done with Chelex, Chelating Ion Exchange Resin (BioRad Labs, Hercules, CA, USA), as described previously (26). Two amplification reactions were performed as semi-nested PCR, each with three different primers (Eurobio) suitable for hybridization within the conserved regions of genes coding for outer membrane protein A (ompA) and citrate synthase (gltA), respectively (Table 2; ref. 5,27,28).

Table 2. Oligonucleotide primers used for semi-nested polymerase chain reaction (PCR) amplification of the DNA extracted from culture isolate

Primer	Nucleotide sequence (5'-3')	Position of	Fragment size (bp)
rOmpA:		ompA	
190-70	ATG GCG AAT ATT TCT CCA AAA	70-90	630/520
190-180	GCA GCG ATA ATG CTG AGT	149-166*	
190-701	GTT CCG TTA ATG GCA GCA TCT	701-681*	
gltA:		citrate synthase	
AF7F	TGA TTC AGA ATT TGC TGA ATT	1-20	270/220
AF8F	ATT ACC TAT ACT TAA AGC AAG	46-66*	
PUL6R	AAT TAT TAT ACT GCT CGC CA	308-289*	

*Primers used for sequencing.

The success of the amplification was confirmed by resolution of the products by electrophoresis on a 1% agarose gel for products of ompA gene, and on a 2% gel for products of citrate synthase gene. The sizes of the PCR-amplified products were determined by comparison with a molecular weight standard (marker VI and V for ompA and citrate synthase gene, respectively) under the ultraviolet light following ethidium bromide staining.

Sequencing Reactions and Data Analysis

The PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's recommendations. Sequencing reactions were carried out with a

DNA sequencing kit, dRhodamine Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on an ABI PRISM 310 DNA Sequencer (Applied Biosystems).

The obtained sequences were identified by comparison with sequences available in GenBank by using the BLAST software (29).

Serology

The sera were tested for the presence of IgM- and IgG-specific antibodies by the indirect immunofluorescence assay (IFA) using commercially available antigen slides (*Rickettsia conorii*-spot IF assay; bioMerieux, Lyon, France), according to the manufacturer's instructions. The titers of IgM and IgG were determined in acute- and convalescent-phase serum samples diluted twofold in phosphate buffered saline (PBS) beginning at 1:20. Sera found positive (IFA titer > 1:40) were titrated to the endpoint. Positive titer of IgM and IgG antibodies only in convalescent-phase serum was considered seroconversion.

To remove IgG, rheumatoid factor absorbent was used prior to IgM determination, according to the manufacturer's instructions (RF-absorbent; Behring AG, Marburg, Germany).

Results

Rickettsiae were detected by PCR and culture in only a single out of 17 patients with Mediterranean spotted fever. PCR primers used in the "suicide" PCR for the genus-specific ompA gene amplified a fragment of the expected size from the blood sample, but the amount of amplified DNA was not sufficient for sequencing (Fig. 1).

Moreover, six days after the inoculation of the patient's blood into the shell vials, the Gimenez staining of the HEL cells revealed short coccobacillary forms, retaining basic fuchsin, whereas the indirect immunofluorescence on the cover slip gave a remarkably positive result (Fig. 2).

After PCR procedure with the rickettsial isolate, the desired genes' parts were amplified (Figs. 3 and 4). The sequences of the ompA and citrate synthase gene, respectively, derived from the shell vial isolate found to share 100% similarity with the ompA (GenBank accession number U43806) and citrate synthase (GenBank accession number U59730) genes of *Rickettsia conorii*, strain Seven (Malish) (5,6,27,28).

Serological testing of acute phase sera gave negative results for IgM and IgG antibodies (titers < 1:40).

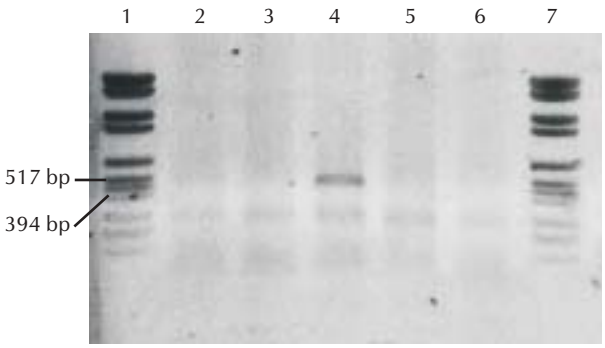

Figure 1. Agarose gel stained with ethidium bromide showing the amplified ompA fragment obtained from a patient's blood DNA after "suicide" polymerase chain reaction (lanes 4 and 5 are the patient's amplicons, concentrated and diluted 1:10, respectively). Molecular weight markers are in lanes 1 and 7. Lane 6 is a negative control.

Four patients did not return for a follow-up visit, so the diagnosis remained presumptive, based solely on clinical and epidemiologic data. In the remaining 13 patients, including the patient with positive isolation

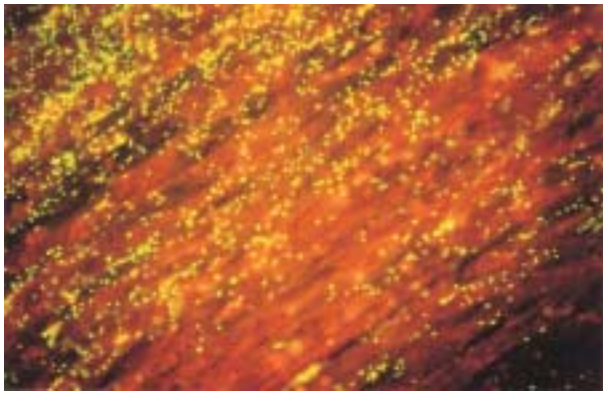

Figure 2. Photomicrograph showing multiplication of *Rickettsia conorii* in human embryonic lung (HEL) cells from the shell vial culture (indirect immunofluorescence staining, magnification ×400).

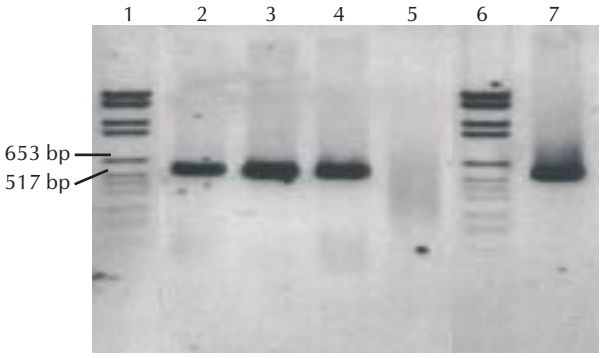

Figure 3. Agarose gel stained with ethidium bromide showing amplified fragment of ompA gene from the culture isolate (lanes 2, 3, and 4 representing amplified products concentrated, diluted 1:10, and diluted 1:100, respectively). Lanes 1 and 6 are molecular weight markers. Lanes 5 and 7 are a negative and positive control, respectively.

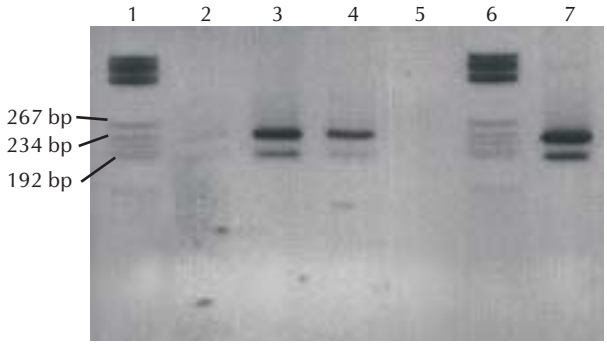

Figure 4. Agarose gel stained with ethidium bromide showing amplified fragment of citrate synthase gene from the culture isolate (lanes 2, 3, and 4 representing amplified products concentrated, diluted 1:10, and diluted 1:100, respectively). Lanes 1 and 6 are molecular weight markers. Lanes 5 and 7 are a negative and positive control, respectively. Large amount of nucleic acid in the concentrated mixture prevented the proper amplification of the target DNA (no band in lane 2).

of *R. conorii*, paired sera were obtained and seroconversion confirmed.

Discussion

Laboratory diagnosis of Mediterranean spotted fever is usually determined by serological testing. However, during the first days of the disease, when antibodies are not detectable, rapid diagnosis should be based on the detection of the rickettsiae in clinical samples. Rickettsiae can be detected and identified by immunohistochemical staining, PCR, and isolation (1,30).

The isolate in this report was identified as *Rickettsia conorii*. To the best of our knowledge, this is the first isolation of *R. conorii*, the causative agent of Mediterranean spotted fever in Croatia, obtained from a human blood sample. Although we performed the "suicide" PCR, known for its high sensitivity (25,30,31), the obtained minor band in only one blood sample and the negative results for the rest of them could be explained by the difficulties often encountered with PCR on blood (30-33), as well as by the possible nucleic acid changes caused by the freezing and thawing of the sample. It is also possible that there were too few circulating rickettsiae in the blood at the time when the sample was taken (32). A better result might be achieved if the buffy coat was taken before freezing the blood samples, because thus it would contain more endothelial cells and rickettsiae, respectively, and less inhibitors (30,33).

Nevertheless, the shell vial culture of the blood sample of the same patient was a powerful tool in identifying the causative agent of the patient's illness, regardless of the fact that the blood had been frozen for more than two years before inoculation. Although rickettsial isolation takes considerably longer time than PCR detection, which, if positive, provides a rapid and reliable diagnosis, it was proven as method of choice in establishing rickettsial isolate with further identification and propagation of the strain (1,23,34).

In this study, the final diagnosis of Mediterranean spotted fever in the majority of patients was established serologically, by using IFA, which is still considered as a test of choice for the serodiagnosis of rickettsial diseases (30). The known disadvantage of the commercially available IFA reagent kits is cross-reaction between species within the rickettsial biogroup, which does not allow correct species identification (1,20,30). Multiple microimmunofluorescence assays with different species antigens or a combination of Western-blot and cross-absorption technique are thus required (1,30), but still available only in reference laboratories.

This is the first report where the role of *Rickettsia conorii* in Mediterranean spotted fever in Croatia was confirmed by culture, a classical microbiological method.

We hope that this study will raise microbiologists' and clinicians' awareness and recognition of Mediterranean spotted fever rickettsioses. Previous reports on malignant forms of Mediterranean spotted fever and a recent description of the first fatal case in Croatia (35-38) accentuate the need for prompt diagnosis. Therefore, diagnostic methods should be improved and rickettsiae and their vectors further investigated in this endemic region of south Croatia.

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