ORIGINAL ARTICLE 10.1111/j.1469-0691.2008.02013.x

# Comparison of PCR methods and culture for the detection of *Borrelia* spp. in patients with erythema migrans

*T. Cerar[1], E. Ružić-Sabljić[1], U. Glinšek[1], A. Zore[2] and F. Strle[3]*

[1]Institute of Microbiology and Immunology, Faculty of Medicine, [2]University of Ljubljana, College of Health Studies and [3]Department of Infectious Diseases, University Medical Centre Ljubljana, Ljubljana, Slovenia

## ABSTRACT

The sensitivities of two PCR assays and culture were compared for the detection of *Borrelia* spp. in skin specimens of 150 patients with typical erythema migrans. In addition, the accuracy of the methods for the identification of *Borrelia* spp. was compared by analysing culture isolates and material obtained directly from skin biopsy specimens. *Borrelia burgdorferi* sensu lato was isolated from 73 (49%) of 150 skin biopsy specimens. Using a nested PCR targeting the *rrf–rrl* region and a PCR targeting the flagellin gene, 107 (71%) and 36 (24%) specimens, respectively, were positive. With both PCRs, positive results were more frequent with culture-positive samples (67/73 (92%) and 24/73 (33%) for the nested and flagellin PCRs, respectively) than with culture-negative samples (40/77 (52%) and 12/77 (16%) for nested and flagellin PCR, respectively). Pulsed-field gel electrophoresis after *Mlu*I restriction identified 69/73 (95%) isolates, of which 58/69 (84%) were *Borrelia afzelii* and 11/69 (16%) were *Borrelia garinii*. After *Mse*I restriction of PCR products amplified from the intergenic *rrf–rrl* region, *B. afzelii* was identified in 73/107 (68%) samples, *B. garinii* in 22/107 (21%) samples, and both species in 11/107 (10%) samples. The corresponding results for culture-positive specimens were 41/69 (59%), 14/69 (20%), and 7/69 (10%). Comparison of the results for specimens positive according to both approaches revealed complete uniformity in 80% of the cases. Overall, nested PCR was the most sensitive method for the demonstration of *Borrelia* spp. in erythema migrans skin lesions, followed by culture and PCR targeting the flagellin gene. The congruence of identification results obtained by analyzing culture isolates and material obtained directly from skin biopsies was relatively high but incomplete.

**Keywords** *Borrelia* spp, cultivation, erythema migrans, identification, PCR, skin biopsies

**Original Submission:** 3 October 2007; **Revised Submission:** 21 December 2007; **Accepted:** 14 February 2008

Edited by P. Brouqui

*Clin Microbiol Infect* 2008; **14:** 653–658

## INTRODUCTION

Lyme borreliosis is a tick-borne zoonosis caused by *Borrelia burgdorferi* sensu lato. Within the *B. burgdorferi* complex, at least three species are known to cause the disease in humans, i.e. *Borellia afzelii*, *Borrelia garinii* and *B. burgdorferi* sensu stricto [1]. Lyme borreliosis is a multisystemic disease. Its initial manifestation is usually represented by a cutaneous lesion named erythema migrans [2,3]. In Slovenia, as well as in the majority of European countries, Lyme borreliosis is endemic, and erythema migrans is by far the most frequent clinical manifestation [3] (http://www.ivz.si/javne_datoteke/datoteke/798-Epidemiolosko_spremljanje_NB_2006.pdf). When typical, this skin lesion provides a reliable diagnosis of Lyme borreliosis, and can serve as a reference standard for the evaluation of microbiological tests.

Several different microbiological approaches have been used to confirm borrelial infection, including isolation of *Borrelia* spp. from clinical specimens, detection of borrelial DNA by PCR, and detection of specific antibodies in body fluids [3–5]. Serological tests are often negative in patients with erythema migrans, while direct detection methods, e.g. culture or PCR, are positive more frequently [6–8]. Culture of *Borrelia* spp. is the reference standard for demonstration of borrelial infection, but has relatively low sensitivity (the highest

Corresponding author and reprint requests: T. Cerar, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1105 Ljubljana, Slovenia
E-mail: tjasa.cerar@mf.uni-lj.si

sensitivity of 50–70% being obtained with skin biopsy specimens of typical erythema migrans lesions), and is technically demanding and time-consuming. The main advantages of PCR are its higher sensitivity and faster performance [3–5].

In the present study, two PCR assays (targeting the flagellin gene and the intergenic *rrf–rrl* spacer, respectively) were compared with culture for the detection of *Borrelia* spp. in skin biopsy specimens from patients with erythema migrans. The efficacy and congruence of methods for the identification of *Borrelia* spp. were also compared by typing culture isolates and material obtained directly from skin biopsy specimens.

## MATERIALS AND METHODS

### Patients

Biopsy specimens of skin lesions were obtained from 150 patients diagnosed with typical, previously untreated, erythema migrans, who presented at the Department of Infectious Diseases, University Medical Centre, Ljubljana. The study was approved by the Medical Ethic's Committee of the Republic of Slovenia. Of the 150 samples, 144 had been used previously in the study described by Zore *et al.* [9], but the aims and approaches of the two studies differed. Typical erythema migrans was defined according to CDC criteria [10]. In addition, patients with skin lesions <5 cm in diameter were also included if they recalled a recent tick bite at the site of the skin lesion, had a symptom-free interval between the bite and the onset of the lesion, and reported an expanding skin lesion before diagnosis.

### Skin specimens

A skin biopsy specimen measuring 5 × 2 × 2 mm was taken from the periphery of the site of erythema migrans after disinfection with alcohol 70% (v/v) and local anaesthesia with xylocaine 2% (w/v) [11]. Each biopsy specimen was dissected into two equal parts: one was immediately inoculated into modified Kelly–Pettenkofer medium and promptly transported to the laboratory; the other was frozen at −70°C for PCR analysis. Cultivation and PCR analysis were performed at the Institute of Microbiology and Immunology of the Faculty of Medicine, Ljubljana.

### Cultivation

Skin biopsy specimens (*c.* 2.5 × 2 × 2 mm) were incubated in modified Kelly–Pettenkofer medium at 33°C, and were examined at weekly intervals by dark-field microscopy. Samples were considered to be negative if no growth was detected after incubation for 9 weeks [12].

### Nucleic acid isolation from skin samples

Nucleic acid was isolated from skin biopsy specimens (*c.* 2.5 × 2 × 2 mm) that had been stored at −70°C. Each biopsy specimen was dissected into small pieces, and incubated overnight at 56°C with proteinase K and lysis buffer; this was followed by isolation of the DNA using a QIAamp tissue kit (Qiagen, Santa Clara, CA, USA), all according to the manufacturer's instructions. Isolated DNA was stored at −20°C until further analysis.

### PCR

Primers targeting two different genes were used. Primers for amplification of the flagellin gene [13] were used under the conditions described by Zore *et al.* [14]. Amplicons of 289 bp were visualized on agarose (3% w/v) gels stained with ethidium bromide. PCR amplification of the intergenic *rrf–rrl* region was performed using primers described by Postic *et al.* [15]. In brief, 10 μL of isolated DNA was amplified with external primers SPA1 and SPA2 and internal primers P1 and P2 using 20 cycles of 3 min at 93°C, 2 min at 70°C and 2 min at 72°C, and then 40 cycles of 1 min at 93°C, 2 min at 50°C and 2 min at 72°C, followed by a 7-min hold at 72°C. Amplicons (250 bp) were detected on agarose (3% w/v) gels stained with ethidium bromide.

The quality of each DNA sample was verified by amplification of a 268-bp fragment of the human β-globin gene using primers PC04 and GH20 [16]. In addition, a panel of positive and negative control samples was included in each experiment to monitor amplification and contamination. Strict precautions were also taken to avoid PCR contamination and amplicon carryover, with processing of PCR samples performed in separate rooms and the use of filter pipette tips [17].

### Identification of *Borrelia* spp.

*Genotypic characterisation of PCR-positive skin biopsy specimens.* Nested PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis with 5 U of *Mse*I (New England Biolabs, Ipswich, MA, USA), as described by Postic *et al.* [15]. *Mse*I-digested fragments were visualized by electrophoresis on acrylamide 16% (w/v):bisacrylamide 0.8% (w/v) gels for 2 h at 110 V, and staining with ethidium bromide [15]. RFLP patterns of samples were compared with RFLP patterns of control *B. burgdorferi sensu lato* spp. [15].

*Genotypic characterisation of Borrelia isolates.* DNA from borrelial isolates was extracted by the gel-insert method for pulsed-field gel electrophoresis (PFGE) analysis, and by using a QIAamp DNA Mini Kit (Qiagen) for PCR-based RFLP analysis, as described previously [12,15]. For PFGE, samples were digested with *Mlu*I (New England Biolabs) at 37°C for 24 h. Restriction fragments were separated using ramped pulse times of 1–40 s for 24 h, as described previously [18]. Identification of a particular species was based on an analysis of RFLP patterns, with bands at (i) 440, 320 and 90 kb, (ii) 220 and 80 kb and (iii) 145 bp being interpreted as specific for *B. afzelii*, *B. garinii* and *B. burgdorferi sensu stricto*, respectively [12,19–21]. For PCR-based genotyping, *rrf–rrl* amplification products were subjected to RFLP analysis after restriction with 5 U of *Mse*I, as described for the characterisation of PCR-positive skin samples [15].

### Statistical analysis

Yates' corrected chi-squared test, with the level of significance set at $p < 0.05$, was used for statistical comparisons.