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

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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

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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

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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.

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RESULTS

B. burgdorferi sensu lato was isolated from 73 (48.7%) of 150 skin biopsy specimens. Using nested PCR targeting the *rrf–rrl* region, 107 (71.3%) skin biopsy specimens were positive, while PCR targeting the borrelial flagellin gene was positive for 36 (24%) skin biopsy specimens (Table 1). Both PCR approaches yielded positive results more frequently with culture-positive than with culture-negative patients; 67 (91.8%) culture-positive and 40 (51.9%) of 77 culture-negative specimens were positive according to *rrf–rrl* PCR ($p < 0.0001$), while the corresponding figures for the flagellin-based PCR were 24 (32.9%) and 12 (15.6%), respectively ($p = 0.0222$). The two PCR tests gave concordant results in 79 (52.7%) cases—36 samples were positive and 43 samples were negative according to both tests. All samples that were positive according to the flagellin PCR were also positive according to the nested *rrf–rrl* PCR (Table 2). No PCR inhibition was observed.

Characterization of the isolated strains by PFGE enabled the identification of 69 (94.5%) of 73 skin isolates; the remaining four isolates grew too slowly to enable further analysis by PFGE (Table 3). PFGE genotyping identified *B. afzelii* and *B. garinii* in 58 (84%) and 11 (16%) specimens, respectively; no *B. burgdorferi sensu stricto* was identified. Genotyping results according to PCR-based RFLP were in complete agreement with the results according to RFLP based on PFGE. Four 'slow' isolates could be identified only to the species level by PCR-based RFLP analysis: two were identified as *B. afzelii*, one as *B. garinii*, and one as a mixture of *B. afzelii* and *B. garinii* (Table 3). PCR-positive skin biopsy specimens ($n = 107$) were genotyped using PCR-based restriction with *Mse*I. *B. afzelii* was identified in 73 (68.2%), *B. garinii* in 22 (20.6%) and mixed infections with both *Borrelia* species in 11 (10.3%) biopsy specimens. One (0.9%) biopsy specimen yielded a non-specific restriction pattern (currently undergoing further sequence analysis), but no *B. burgdorferi sensu stricto* was identified (Table 3). The corresponding results for the 69 culture-positive specimens were 41 (59.4%), 14 (20.3%), 7 (10.1%) and none for *B. afzelii*, *B. garinii*, mixed infections, and non-specific restriction pattern, respectively.

Analysis of the 69 culture-positive specimens revealed that the two approaches for genotypic characterization (typing culture isolates and typing material obtained directly from skin biopsy specimens) yielded identical results for 48 (69.6%) of the samples (Table 3). Of the 21 (31.4%) discrepant samples, six (8.7%) did not contain detectable borrelial DNA in the skin biopsy specimens, seven (10.1%) yielded different species identifications according to the two methods, and another seven (10.1%) revealed the presence of two *Borrelia* species according to PCR-based RFLP of material obtained directly from skin, while PFGE, as well as PCR-based RFLP of the actual isolates, indicated the presence of only one *Borrelia* species (Table 3).

Table 1. Comparison of results obtained using two PCR methods and culture for the demonstration of borrelial infection in patients with erythema migrans

	PCR			
	Nested *rrf–rrl*		Flagellin gene	
Culture	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Positive (n = 73)	67 (91.8)	6 (8.2)	24 (32.9)	49 (67.1)
Negative (n = 77)	40 (51.9)	37 (48.1)	12 (15.6)	65 (84.4)
All (n = 150)	107 (71.3)	43 (27.7)	36 (24)	114 (76)
p-value	<0.0001		0.0222	

Table 2. Comparison of results obtained with the PCR targeting the intergenic *rrf–rrl* region and the PCR targeting the flagellin gene

	Flagellin gene		
	Positive	Negative	All
Nested *rrf–rrl*			
Positive	36	71	107
Negative	0	43	43
All	36	114	150

Table 3. Comparison of the identification results obtained using pulsed-field gel electrophoresis of *Mlu*I-digested DNA from cultured skin isolates and PCR-based restriction fragment length polymorphism (RFLP) analysis of DNA obtained from skin biopsy specimens

	Cultured isolates				
	Positive—*Mlu*I restriction				
Skin biopsies	Ba (n = 58)	Bg (n = 11)	Ng[a] (n = 4)	Negative	All
Nested PCR (*rrf–rrl*)					
Positive—*Mse*I restriction					
Ba	40	1	2	30	73
Bg	6	8	1	7	22
Ba + Bg	6	1	1	3	11
Non-typeable	1	0	0	0	1
Negative	5	1	0	37	43

Ba, *Borrelia afzelii*; Bg, *Borrelia garinii*; Ng, no growth.
[a]Isolates identified to species level by PCR-based RFLP analysis.

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DISCUSSION

Typical erythema migrans is a manifestation of Lyme borreliosis that allows a reliable clinical diagnosis of the disease; when erythema migrans is present, microbiological confirmation of the diagnosis is usually not required. However, patients with erythema migrans form an interesting group in which to assess the sensitivity of microbiological approaches for demonstrating borrelial infection. The present study therefore compared two PCR methods and culture for identifying *Borrelia* spp. in untreated patients with typical erythema migrans. The study used almost the same material as that employed by Zore *et al.* [9]; however, nested PCR targeting the intergenic *rrf–rrl* region was used, which enables identification of individual *Borrelia* spp. Genotyping according to PFGE following *Mlu*I restriction of intact chromosomal DNA was compared with the findings of the nested PCR, following restriction with *Mse*I. In addition, the genotyping results with *Borrelia* isolates obtained from skin were compared with the results obtained following direct detection in skin biopsy specimens.

B. burgdorferi sensu lato was isolated from 48.7% of skin biopsy specimens. This isolation rate was lower than that reported in some previous studies utilising analogous methodology [9,22–24]. The sensitivity of culture depends upon many factors, including the medium, techniques employed, source of specimens, and the quality and quantity of *Borrelia* organisms present in the samples [3–5,22–25]. The rather small samples obtained for culture in the present study might explain, at least in part, the relatively low yield. Although a medium without antibiotics was used, cleansing of skin with alcohol 70% (v/v) before performing a skin biopsy, and the use of xylocaine 2% (w/v) as a local anaesthetic, could have had detrimental effects on the recovery of *Borrelia* organisms from skin biopsy specimens.

The present study revealed that nested PCR amplification of the intergenic *rrf–rrl* region was found to be more sensitive than amplification of the flagellin gene (71.3% vs. 24% PCR-positive samples, respectively). The results of the two PCR assays were in accord in only 52.7% of cases, but all flagellin gene-positive specimens were also positive using nested PCR. The finding that nested PCR is more sensitive than flagellin-based PCR has been reported previously [4,5,9,26], and is in agreement with the reported *in vitro* sensitivity of 1–10 bacteria/mL for PCR targeting the intergenic *rrf–rrl* region and 100–1000 bacteria/mL for the flagellin PCR [14,27]. With the nested PCR, borrelial DNA was detected in all but six (67/73, 91.8%) of the culture-confirmed samples. The fact that not all culture-positive specimens were also PCR-positive could be explained by a low number of spirochaetes in individual skin biopsy specimens, their unequal distribution, and/or the presence of inhibitors in samples, while even a few organisms in a skin biopsy specimen can multiply in culture during the incubation time of several weeks [28,29]. All samples in the present study (including those that were PCR-negative) were positive for the amplification of the human β-globin gene, indicating that a low number of *Borrelia* organisms in the samples, rather than inhibition of the PCR, was probably a primary cause of negative PCR results. Theoretically, discrepancies between culture and PCR could also be explained by laboratory contamination, although strict measures were taken to prevent this occurring.

PCR targeting the intergenic *rrf–rrl* region also has the advantage of the possibility of species identification by means of the PCR product, which also confirms the specificity of the amplified products [1,15]. In comparison with PFGE, PCR-based RFLP also enabled the direct detection and species identification of borreliae present in skin biopsy specimens. PCR-based identification is also faster, is less expensive and requires less preparation of the sample. Thus, fewer *Borrelia* cells are needed for PCR-based identification than for PFGE, as demonstrated with the four isolates that were typed by PCR only because they grew too slowly to allow the use of PFGE.

Most isolates in the present study were identified as *B. afzelii*; *B. garinii* was also detected, but *B. burgdorferi* sensu stricto was not recognized. These findings confirm the results of previous studies in Slovenia, in which *B. afzelii* was the most common aetiological agent of erythema migrans, i.e. in 75–89% of the cases [8,9,12,20,24]. The results according to PFGE and PCR-based identification were in complete agreement, although several differences were found when comparing genotyping results for the actual *Borrelia* isolates with those for material obtained directly from skin. Not only was the sensitivity of the nested PCR for direct detection of *Borrelia* in

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skin biopsy specimens substantially higher than that of culture, but some differences were found regarding the identification of the species causing skin lesions. Analysis of a subgroup of isolates from 69 culture-positive specimens characterized by PFGE revealed that 21 (31.4%) of the samples yielded different, method-dependent results. The demonstration of mixed *Borrelia* infections directly in skin samples, but not in culture, has been reported previously and has been interpreted as a result of overgrowth of one species by another during culture [26,30]. Thus, identification of *Borrelia* spp. by PCR using skin material offers more comprehensive information about the precise species responsible for infection.

Cultivation of *Borrelia* isolates and their typing by PFGE remains a reference standard for the demonstration of borrelial infection [3,5,12]. However, this approach provides rather delayed results and is technically demanding and time-consuming. The main advantages of PCR for the demonstration of a *Borrelia* infection are its enhanced sensitivity and speed, coupled with the possibility of performing the analysis directly on the original biopsy specimen.

In conclusion, nested PCR targeting the intergenic *rrf–rrl* region had the highest sensitivity (71.3%) for detecting *Borrelia* in skin biopsy specimens of patients with erythema migrans, followed by culture (48.7%) and flagellin PCR (24%). All flagellin PCR-positive specimens, as well as most of the culture-positive cases, yielded positive results with the nested PCR targeting the intergenic *rrf–rrl* region. While the species identifications of the actual isolates, as determined by PFGE and nested PCR, were in complete agreement, different results were obtained in several cases when identifications were made with either bacterial isolates or skin biopsy specimens as the starting material.

TRANSPARENCY DECLARATION

The authors declare that they have no conflicting interests in relation to this work.

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