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Amplification of *Borrelia burgdorferi* DNA in Skin Biopsies from Patients with Lyme Disease

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To determine whether the polymerase chain reaction could contribute to a better diagnosis of Lyme disease, skin biopsy samples from patients suffering from erythema chronicum migrans or acrodermatitis chronica atrophicans were tested for the presence of *Borrelia burgdorferi* by a polymerase chain reaction assay, which was specific for European strains. The spirochete could not be detected microscopically in any of the 15 biopsy samples obtained from nine patients. However, *B. burgdorferi* could be isolated from seven of eight of these samples, which indicated the presence of spirochetes. Using a nested polymerase chain reaction, we were able to detect *B. burgdorferi*-specific sequences in 12 of the 15 biopsy samples. Biopsy samples from three of four patients with erythema chronicum migrans and four of five patients with acrodermatitis chronica atrophicans were found to be positive for *B. burgdorferi*. The spirochete could be isolated from the biopsy sample, from a patient with erythema chronicum migrans who tested negative, which suggests a false-negative polymerase chain reaction result probably on account of the low number of spirochetes present in the lesion. The positive polymerase chain reaction for lesions from patients with acrodermatis chronica atrophicans supports the concept that *B. burgdorferi* can persist in the skin over a long period of time. From these results, it was concluded that the polymerase chain reaction is a valuable technique for the diagnosis of Lyme disease.

Lyme borreliosis has been reported with increasing frequency during the last several years, and it is now the most common tick-borne infection in Europe and the United States (7, 8, 28).

Borrelia burgdorferi is transmitted to humans and other vertebrates by infected ticks of the *Ixodes ricinus* complex (6). It may cause a variety of different clinical signs. In the initial phase, a characteristic distinctive skin lesion, erythema chronicum migrans (ECM), can be observed in up to 60% of the patients (28). The initial phase of the disease is benign and can be treated with antibiotics (32). Later stages of Lyme disease may be concomitant with progressive encephalomyelitis, chronic arthritis, and acrodermatitis chronica atrophicans (ACA), a late skin manifestation of this disease which is mainly found in Europe (1, 3, 29, 33). This chronic phase is difficult to treat. In order to prevent the development of severe late manifestations of Lyme disease, an early diagnosis is essential.

It is difficult to make a reliable diagnosis of Lyme disease on clinical grounds alone, because of the variations in its presentation (29). Isolation of *B. burgdorferi* from patient specimens is difficult, and microscopic detection of the spirochete is not very sensitive (4, 11). Therefore, serology is most widely used to diagnose this disease (10, 16). However, antibodies against *B. burgdorferi* can be detected only in 40 to 60% of the patients during the first 3 to 6 weeks of infection or in patients with ECM (13, 15). These limitations emphasize the need for rapid, reliable, and sensitive methods for the diagnosis of Lyme disease.

Recently, attention has focused on the polymerase chain reaction (PCR) as an aid in diagnosis (17, 22–27). By using this in vitro amplification technique, it appeared to be possible to detect as few as 10 spirochetes per ml of blood or urine (17). However, the PCR has been used only for the detection of *B. burgdorferi* in ticks or model systems, and the usefulness for the detection of the spirochete in human specimens remains to be established.

To determine the value of the PCR as a diagnostic tool for Lyme disease, a specific European PCR assay was used (25) for the detection of *B. burgdorferi* in skin biopsy specimens from Dutch patients who were clinically suspected of having Lyme disease.

MATERIALS AND METHODS

Patients. Punch biopsy specimens were obtained from patients attending the Department of Dermatology, University of Nijmegen, and Maasland Hospital, Sittard, with a history and skin manifestations suggestive of Lyme disease, as indicated in Table 1. Also included were a biopsy sample from a patient with morphea and a tick which was *B. burgdorferi* negative by conventional methods.

As negative controls, biopsy specimens from a patient with a plasmacytoma and human spleen were incorporated. Biopsy specimens were stored and frozen (−80°C) for periods that varied from several months to years until the DNA was extracted for PCR.

***B. burgdorferi* isolates.** The *B. burgdorferi* isolates used as positive controls originated from the United States (tick), isolate ATCC/p35; Germany (skin), isolates PKO/p22, PKO/p33-7p2, and PKO/p33-42; and The Netherlands (tick), isolates M18 and M06.

Cultivation of *B. burgdorferi*. Immediately after the biopsy specimens were obtained, the tissues, which were similar in size between the different patients, were put in BSK II

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TABLE 1. Specifications of patients examined in this study

Sample no.[a]	Patient		Dermatological diagnosis		Localization	Lyme serology reciprocal titer[c]		*B. burgdorferi* detection[d]		Treatment[e]
	Sex[b]	Age (yr)	Lesion	Duration		IgG	IgM	Culture	PCR	
1a	F	70	ECM	3 yr	Upper leg edge	N	N	ND	−	Doxycycline, 30 days at 200 mg/day
1b					Upper leg edge	N	N	ND	+	
2	M	30	ECM	10 mo	Upper scapula	N	N	+	−	Doxycycline, 15 days at 200 mg/day
3a	F	73	ECM	6 wk	Right arm center	128	64	+	+	Ceftriaxone, 15 days at 3 g/day
3b					Right arm edge	128	64	+	+	
3c					Knee center	128	64	−	+	
3d					Knee edge	128	64	+	+	
4a	F	50	ECM	3 mo	Right leg center	126	N	−	+	Doxycycline, 15 days at 200 mg/day
4b					Right leg edge	126	N	+	+	
4c					Right leg edge	126	N	+	+	
5	M	37	ACA	10 yr	Upper left leg	>256	32	ND	+	Penicillin G, 20 days at 20×10^6 U/day
6	F	51	ACA	1.5 yr	Lower left arm	>256	<16	ND	+	Penicillin G, 20 days at 20×10^6 U/day
7	F	67	ACA	6 yr	Upper right arm	256	<16	ND	+	Penicillin G, 20 days at 20×10^6 U/day
8	M	79	ACA	2 yr	Left foot	1,024	<16	ND	+	Ampicillin, 14 days at 6 g/day, followed by ceftriaxone, 15 days at 3 g/day
9	F	57	ACA	2 yr	Upper left arm	N	ND	ND	−	Not treated

[a] Letters indicate biopsy specimens from different lesions obtained from the same patient.
[b] F, female; M, male.
[c] IgG, immunoglobulin G; N, negative.
[d] ND, not done; +, positive; −, negative.
[e] Treatment after obtaining biopsies.

medium (4) and were transported to the laboratory. The biopsy specimens were divided into two pieces in a laminar airflow hood. Half of the biopsy specimen was used for PCR analysis. The other half was ground and cultured in 5 ml of BSK II medium at 34°C. The medium was examined for spirochetes by dark-field microscopy. Positive cultures were subsequently examined by scanning and transmission electron microscopy. Examination was performed weekly during a period of 3 months until the specimens were regarded negative.

DNA extraction procedures. (i) Biopsy samples. The DNA from the biopsy samples was extracted as described previously (35).

(ii) *B. burgdorferi* strains. The DNA was isolated from *B. burgdorferi* strains by the alkaline-lysis method described by Maniatis et al. (18).

PCR. The PCR was performed essentially as described previously (20, 25, 26). Oligonucleotides were synthesized on a SAM One DNA Synthesizer (Bioresearch, San Raphael, Calif.). Primers (Table 2) of chromosomal origin which are known to amplify DNA from European *B. burgdorferi* strains (25) were used for the single and nested PCR assays.

The preparation of reaction mixtures, the DNA extraction (clinical and positive controls), and the amplification and detection of the PCR products were all performed at different locations. By the recommendations of Kwok and Higuchi (14), this strict spatial partition of the different technical steps involved in PCR is necessary to prevent contamination (20).

Amplification of *B. burgdorferi*-specific target sequences was carried out in a 100-μl reaction mixture containing 250 to 500 ng of DNA from the patient specimens or 1 ng of DNA from the *B. burgdorferi* strains, 50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 200 μM (each) deoxynucleoside triphosphates (i.e., dATP, dTTP, dGTP, and dCTP), 30 pmol of primer sets b/b′ or c/c′ and 2 U of the thermostable *Taq* DNA polymerase (Perkin-Elmer Cetus, Gouda, The Netherlands). The reactions were overlaid with mineral oil (≈100 μl) to prevent evaporation and were subjected to 40 cycles of amplification by using an automated DNA thermal cycler (Perkin-Elmer Cetus). Each cycle involved heating to 94°C for 1 min (DNA denaturation), cooling to 37°C for 30 s (primer annealing), and again heating to 60°C for 1 min (primer extension). Low-stringency conditions were used for optimal amplification (25, 26).

For nested PCR, the first 30 cycles were performed with primer set b/b′ under the conditions described above. After

TABLE 2. *B. burgdorferi* primer sequence specifications

Primer	Sequence	Amplified product (bp)
b	5′-GATAAAAACGAAGATAATCG-3′	356
b′	5′-ACTAGGATCTGTGGATATTC-3′	
c	5′-CCAACTTTATCAAATTCTGC-3′	126
c′	5′-AGGATCTATTCCAAAATC-3′	

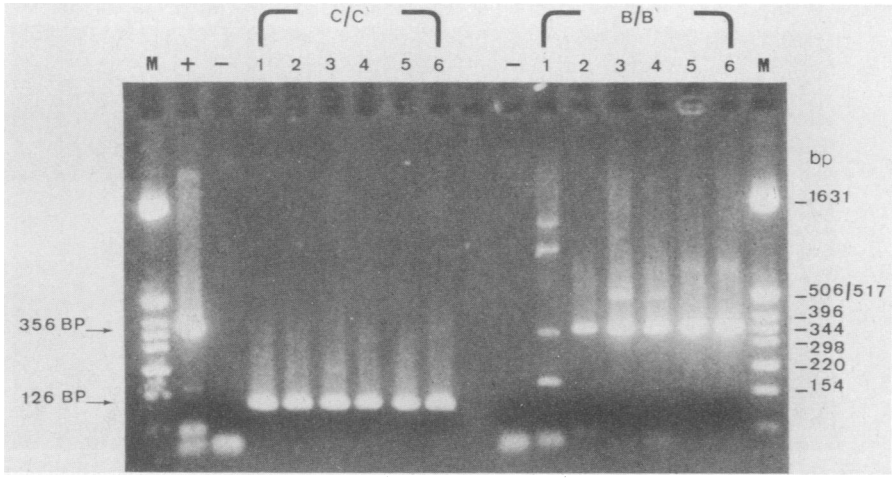

FIG. 1. DNA extracted from different *B. burgdorferi* isolates was tested for specific amplification with the b/b′ and c/c′ primer sets and was examined by agarose gel electrophoresis and ethidium bromide staining. By using the universal *B. burgdorferi* c/c′ primer set, a specific amplification of a 126-bp fragment was observed in both the American isolate ATCC/p35 (lane 1) and the European isolates PKO/p22, PKO/p33-7p2, PKO/p33-42, M18, and M06 (lanes 2 through 6), respectively. By using the European *B. burgdorferi* b/b′ primer set, a specific fragment of 356 bp was generated from all European isolates (lanes 2 through 6), but not from the American isolate (lane 1). M, pBR322 *Hin*fI size standards; +, positive control (American isolate ATCC/p35 amplified by an North American-specific primer set [25, 26]); −, negative control (distilled water).

the last cycle, 5 μl of the reaction mixture was added to a new PCR mixture containing 30 pmol of primer set c/c′, which is directed against the internal portion of the b/b′ amplification product, and was again subjected to an additional 30 cycles. Each cycle for the second reaction in the nested PCR involved heating to 94°C, cooling to 55°C, and heating to 72°C, with an extension of 1 s per cycle. The high-stringency conditions in the nested PCR assay excluded amplification of non-target sequences.

Determination of the PCR amplification products was performed by gel electrophoresis on a 2% agarose gel with ethidium bromide staining and by Southern blot analysis as described previously (20). Hybridization was performed with a probe which consisted of the amplified product of the c/c′ PCR that was separated on low-melting-point agarose (LKB) and that was excised from the gel. DNA was isolated and purified by standard procedures (18). The c/c′-amplified product was labeled with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham International, Buchinghamshire, England) by random prime labeling (Promega).

RESULTS

Sensitivity and specificity of the PCR. To determine the sensitivity of the PCR assay, serial dilutions of purified *B. burgdorferi* DNA were made in the presence of 300 ng of human DNA. By using a "single" PCR, a sensitivity on the gel of 0.1 pg of *B. burgdorferi* DNA was obtained for both the European b/b′ and the general primer set c/c′; this corresponds to approximately 50 genome equivalents. By using a "nested" PCR assay, a sensitivity on the gel of 50 fg of target DNA could be obtained. Hybridization resulted in an increase in sensitivity to less than 10 *B. burgdorferi* genome equivalents.

The specificity of the primer sets for the detection of European *B. burgdorferi* strains was determined by the amplification of an American and five different European strains by using both the b/b′ and c/c′ primer sets. By using the c/c′ primer set, a specific fragment of 126 bp was generated in both the American and European strains (Fig. 1). Amplification with the b/b′ primer set resulted in a specific fragment of 356 bp in all the European strains but not in the American ATCC strain (Fig. 1). As described previously (25), the b/b′ primer set can be considered European *B. burgdorferi* specific, while the c/c′ primer set has a broader specificity. The c/c′ primers are directed against the internal portion of the b/b′-amplified product and can therefore be used as nested primers in two sequential PCRs, to increase the sensitivity of the assay, as described above.

PCR for *B. burgdorferi* detection in patient samples. It was possible to obtain fresh skin biopsy samples from three patients with ECM (patients 2, 3 and 4, Table 1). In vitro cultivation of these biopsy samples resulted in the isolation of *B. burgdorferi* from seven of eight samples (Table 1). A single PCR with the European b/b′ primer set on the DNA extracted from these isolates and on the DNA extracted from tissue biopsy specimens from all patients involved in this study (Table 1) is shown in Fig. 2. This single PCR resulted in the specific amplification of a 356-bp fragment from the European control strain (Fig. 2, lane 2) and the *B. burgdorferi* isolates from patient 3 (Fig. 2, lane 16) and patients 2 and 4 (data not shown). No specific amplification was seen from the American strain (Fig. 2, lane 1) or the biopsy samples (Fig. 2).

Because *B. burgdorferi* was isolated from biopsy specimens of patients 2, 3, and 4, the negative amplification was expected to be the result of the relatively low sensitivity of the single PCR assay. Therefore, a nested PCR was used, as described above. The results are shown in Fig. 3. A specific amplification product of 126 bp was observed from the European strains (Fig. 3, lanes 2 and 16), from the American strain (Fig. 3, lane 1) (de novo amplification with the nested primers and not from the first set [Fig. 2], with which the strain was not reactive), from all the biopsy samples from patient 3 (Fig. 3, lanes 17 through 20), and from patients 5 and 6 (Fig. 3, lanes 8 and 9). Southern blot hybridization (Fig. 4) confirmed the specific amplification and resulted in the detection of three additional positive samples (Fig. 4,

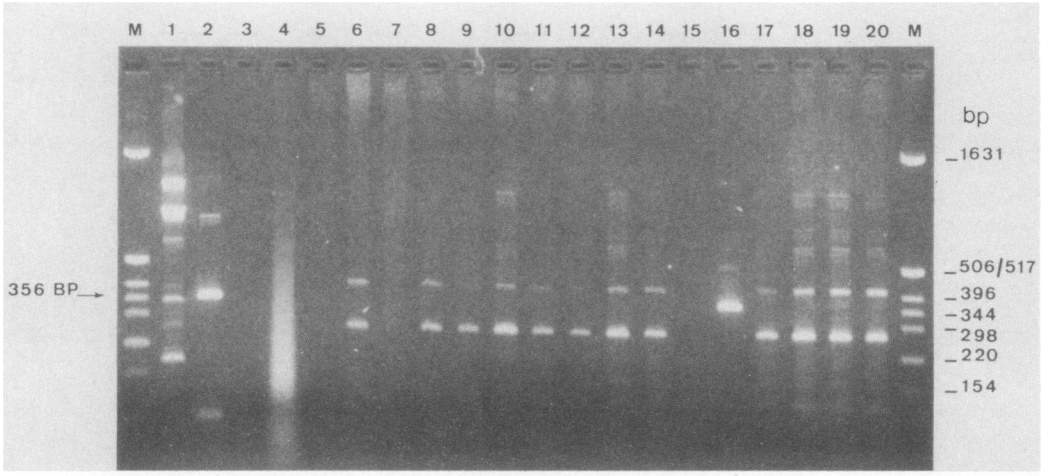

FIG. 2. European-specific *B. burgdorferi* PCR assay was performed by using the b/b′ primer set as described in the text and was examined by agarose gel electrophoresis and ethidium bromide staining. M, pBR322 *Hin*fI size standards. Lane 1, American ATCC/p35 isolate; lane 2, European PKO/p22 isolate; lane 3, distilled water; lane 4, biopsy specimen from a human spleen; lane 5, biopsy specimen from patient 1, specimen a; lane 6, specimen from a patient with plasmacytoma; lane 7, tick; lane 8, biopsy specimen from patient 5; lane 9, biopsy specimen from patient 6; lane 10, biopsy specimen from patient 1, specimen b; lane 11, biopsy specimen from patient 7; lane 12, specimen from a patient with morphea; lane 13, biopsy specimen from patient 8; lane 14, biopsy specimen from patient 9; lane 15, biopsy specimen from patient 2; lane 16, European isolate, patient 3; lane 17, biopsy specimen from patient 3, specimen a; lane 18, biopsy specimen from patient 3, specimen b; lane 19, biopsy specimen from patient 3, specimen c; lane 20, biopsy specimen from patient 3, specimen d. All the stored biopsy specimens (all biopsy specimens except those from patients 2 and 3) were processed in a blind study. Therefore, control specimens (lanes 6 and 7) and biopsy specimens from the same patient (lanes 5 and 10) as well as biopsy specimens from patients with ACA and ECM were processed in a random order. For detailed information about the patient samples, see Table 1.

lanes 10, 11, and 13). The high background in the Southern blot in Fig. 4, lanes 2 and 16 (European *B. burgdorferi* isolates), is a result of the previous amplification with the b/b′ primer set. Although *B. burgdorferi* could be cultured from the biopsy sample from patient 2, no amplification was obtained by PCR. However, amplification was observed from the *B. burgdorferi* strain isolated from this patient. This is in contrast to the results for patient 3, biopsy sample c, which was found to be positive by PCR, but from which no *B. burgdorferi* could be cultured. The results are summarized in Table 1. Of the patients with ECM, three of four were found to be positive for *B. burgdorferi* by PCR. Ten different biopsy samples from those four patients (Table 1) were tested, and eight were found to be positive. Four of five patients with ACA were found to be positive for *B. burgdorferi* by PCR. The spirochete DNA could not be detected in the patient with morphea or in the tick. *B. burgdorferi* could not be detected in the control specimens (plasmacytoma, human spleen, and distilled water; Fig. 3 and 4).

DISCUSSION

In general, it is difficult to diagnose Lyme disease from the clinical picture alone. More than half of the patients suffering from this disease cannot remember a tick bite, and the clinical manifestations are variable. Because of a delay in antibody production in the first 3 to 6 weeks of the disease, serology is not reliable. In addition, *B. burgdorferi* has slow growth properties and is difficult to isolate from infected human tissues (4).

Chronic neurological, cardiological, and dermatological

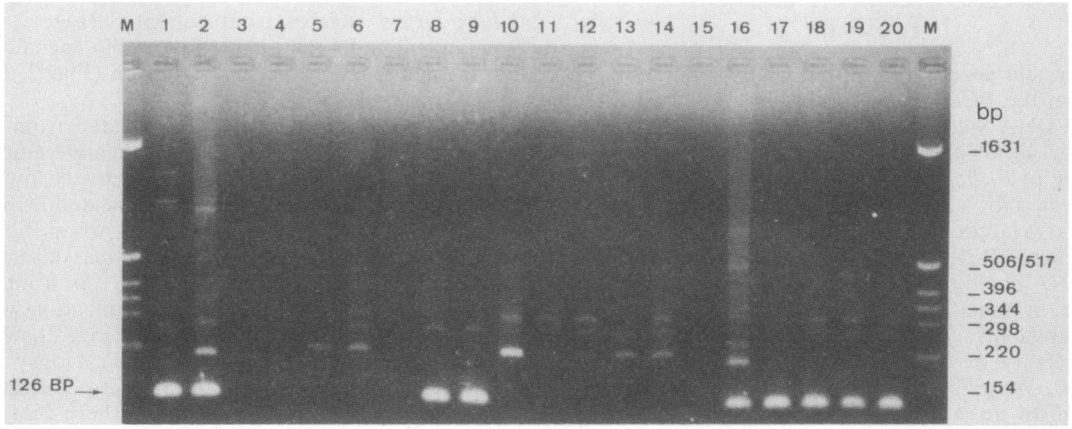

FIG. 3. Nested PCR by using the c/c′ primer set on the European-specific PCR samples (Fig. 2) as described in the text. For lanes and sample specifications, see legend to Fig. 2 and Table 1.

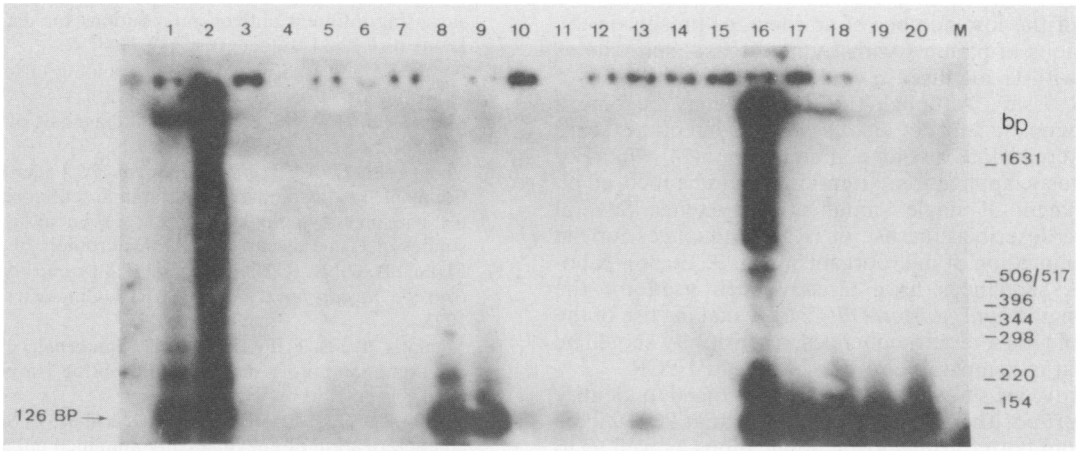

FIG. 4. Southern blot hybridization of the *B. burgdorferi*-specific nested PCR assay shown in Fig. 3. For lanes and sample specifications, see legend to Fig. 2 and Table 1.

disorders may develop when the clinical picture is not recognized in an early stage of Lyme disease.

Several *B. burgdorferi* PCR assays have been developed recently. These assays may lead to a proper diagnosis and to better insights into the pathogenesis of this disease. These assays are based on either chromosomal target sequences or the *ospA* gene of *B. burgdorferi*. Using the *ospA*-based PCR, Persing et al. (24) were able to amplify *B. burgdorferi*-specific sequences from all the ticks that had fed previously on infected animals. They also detected by PCR the spirochete in 10% of museum specimens of *Ixodes dammini* (23). However, the *ospA* gene of *B. burgdorferi* exhibits a broad diversity among the different *Borrelia* strains, and because of this DNA sequence variation, positivity could easily be missed in European strains (25). This emphasizes the need to develop targets for amplification that are more conserved.

In this study, primers directed against chromosomal sequences were used as described by Rosa et al. (25, 26). All five different European isolates could be amplified by the latter PCR assay.

However, this PCR assay failed to detect *B. burgdorferi* in biopsy specimens from patients with skin manifestations of Lyme disease, although spirochetes were isolated from some specimens. This is most probably a result of the relatively limited sensitivity of the test system. A sensitivity of about 50 *B. burgdorferi* genome equivalents can be obtained by a single PCR. It appears that infected humans have a very low density of spirochetes in their tissues. Similar results have been described by Malloy et al. (17). Although they were able to detect 10 organisms per ml of blood or urine in their model system, only 1 of 17 specimens from canines with clinical and serological evidence of Lyme disease was found to be positive by the PCR. They suggested that this negative result was caused by the fact that the samples were taken in a late period of the disease in which the spirochete could not be detected anymore (17). However, it can also be explained by the low number of spirochetes present in the sample (31).

An increase in sensitivity has been described when a nested PCR assay was used for the detection of hepatitis C virus in serum samples (12), and indeed, by a nested PCR assay for *B. burgdorferi*, detection of less than 10 spirochetes appeared to be possible in the model system. Biopsy samples were obtained from four patients with ECM. Three patients were found to be positive for *B. burgdorferi* by PCR. Four different biopsy samples from different lesions from patient 3 and three different biopsy samples from patient 4 were tested for the presence of *B. burgdorferi*. All samples were found to be positive by PCR (Table 1). The location (central or at the leading edge of the lesion) of the biopsy sample (Table 1) did not have an effect on reactivity. Two different biopsy samples from patient 1 were tested. One was found to be positive and one was found to be negative for *B. burgdorferi*. Although *B. burgdorferi* was isolated from the biopsy sample from patient 2, the spirochete could not be detected in this sample by PCR. The isolate, however, was confirmed by PCR to be a European *B. burgdorferi* isolate. Therefore, the negative result of the PCR on these biopsy samples cannot be explained by a divergence in target sequences. It may be caused by sampling error because of the low number of spirochetes in infected tissue. This may also explain the PCR-negative result of the biopsy sample from patient 9, who had ACA. However, this result could also be due to a divergence of the target sequences, as described previously (25).

On the other hand, the spirochete could not be cultivated from two PCR-positive biopsy samples from patients with ECM (patient 3, specimen c, and patient 4, specimen a). This could be either the result of the amplification of nonviable organisms or a sampling error, as described above. Because *B. burgdorferi* could be isolated from the other specimens from these patients, the latter explanation seems more reasonable.

B. burgdorferi has been cultured from lesions of patients with ACA as long as 10 years after the onset of ACA (2), and it can be assumed that *B. burgdorferi* persists in such lesions. *B. burgdorferi* persistence has also been described by Moody et al. (21), who were able to detect the spirochete in different tissues from experimentally induced Lyme borreliosis in rats up until 360 days after infection. This is particularly interesting in view of a possible association of *B. burgdorferi* infection and autoreactive immune responses (29, 30, 34). It is unclear whether *B. burgdorferi* itself is the direct causative agent of ACA, or whether immunological features of the infected patients may play a role in the pathogenesis of this disease. Samples from four of five patients with ACA were positive by PCR. Although positive PCR results are not necessarily correlated with viable organisms, it can be assumed that there are still living spirochetes present in these lesions, indicating *B. burgdorferi* persistence.

Because of the low number of spirochetes present in the different lesions of patients with Lyme disease, spirochetes in patients with Lyme disease could only be identified by a nested PCR assay. Although this method may become a valuable diagnostic tool for *B. burgdorferi* infections, care must be exercised to avoid contamination (14, 20). For future diagnostic application, attention should be focused on the development of single amplification systems. Several reports have described the use of rRNA sequences for the specific amplification of microorganisms (5, 9, 19, 36). Ribosomal DNA sequences have already been used for the amplification of *B. burgdorferi* (17, 23). By making use of the abundance of rRNA target sequences, a sensitivity should be achieved that is comparable to that of a nested PCR.

In this study, we showed that PCR can be used to identify *B. burgdorferi*-positive patients. We believe that PCR will be a valuable tool both for diagnostic applications as well as to gain insight into the transmission of *B. burgdorferi* and the pathogenesis of Lyme disease.

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