Successful Amplification of DNA Specific for Finnish *Borrelia burgdorferi* Isolates in Erythema Chronicum Migrans but Not in Circumscribed Scleroderma Lesions

Annamari Ranki, Einari Aavik, Pärt Peterson, Kristiina Schauman,* and Päivi Nurmilaakso

Departments of Dermatology and Venereal Diseases, and *Serobacteriology, University of Helsinki, Helsinki; Institute of Biomedical Sciences (EA, PP, PN), University of Tampere, Tampere, Finland

Early diagnosis of *Borrelia burgdorferi* infection, hampered by the absence of detectable antibodies in most patients with erythema chronicum migrans is important to prevent late-stage neurologic, rheumatologic, and skin disorders. Furthermore, *B. burgdorferi* has been claimed to be the causative agent in localized scleroderma (morphea). We used PCR amplification to search for *B. burgdorferi* outer surface protein OspA-specific sequences in DNA obtained from lesional skin biopsies on Finnish patients with clinically suspect erythema chronicum migrans, lymphocytoma, morphea, or with diverse skin manifestations and persistent high antibodies to *B. burgdorferi* flagellar antigen. Seronegative patients with other skin lesions served as controls. The amplicons obtained with primers specific for *B. burgdorferi* type strain B31 ospA sequence did not hybridize to the corresponding probes, and thus the DNA amplified from a Finnish *B. burgdorferi* erythema chronicum migrans skin isolate was sequenced. This 98-nucleotide sequence of ospA (332–429) showed 11% to 14% nucleotide divergence compared with the North American type strain (B31), several European strains, and an East Siberian tick strain. The sequence was almost identical (99%) to a Swedish isolate from acrodermatitis chronica atrophicans. Using oligonucleotides specific for the Finnish strain, a positive polymerase chain reaction–based hybridization was obtained in six of seven untreated erythema chronicum migrans patients infected in Finland or in Estonia, and in the lymphocytoma patient. Only two of the erythema chronicum migrans patients had IgG or IgM antibodies to flagellin. However, all seven morphea lesions as well as the other lesions were polymerase chain reaction negative. Polymerase chain reaction–based hybridization of *B. burgdorferi* OspA gene from skin-derived DNA thus provides a sensitive and specific diagnostic tool. In conditions not unequivocally known to be caused by *B. burgdorferi,* like in morphea, this assay was negative. We also demonstrate that peri-Baltic *B. burgdorferi* isolates show homology in their OspA genes but differ from geographically more distant isolates. *Key words: Borrelia burgdorferi/OspA/PCR/erythema chronicum/migrans/morphea. J Invest Dermatol 102:339–345, 1994*

The diagnosis of Lyme borreliosis is frequently impeded by the fact that antibodies against the causative spirochete *Borrelia burgdorferi* are detected only in 30 to 60% of patients during the first 6 weeks of infection, even if the primary skin symptom, erythema (chronicum) migrans (ECM), is present [1–3]. In an Austrian study based on extensive ECM patient material, only 17% and 25% of sera had raised IgM and IgG antibody titers to *B. burgdorferi* [4]. The IgM antibody response is often first seen after a few weeks of antibiotic therapy [5]. Lyme disease shows a complex range of clinical manifestations, from arthritis and neurologic disease to several types of skin lesions [6]. In addition to early-stage ECM, the skin lesions include lymphocytosis cutis benigna, diffuse erythematous lesions, and morphea-like sclerotic skin lesions and acrodermatitis chronica atrophicans (ACA) in the chronic phase [7–9]. In some patients with various other skin lesions, we often see persistently elevated antibodies to *B. burgdorferi,* and the significance and specificity of such antibodies remains to be resolved. Elevated antibody titers have also been demonstrated in localized scleroderma (morphea) [10–11], although contradictory findings have been reported [12–14]. In areas with a high prevalence of *B. burgdorferi* in ticks, a seroprevalence of 4–12%, depending on the method used, is observed in asymptomatic individuals [15,16]. In Finland, the prevalence of positive seroreactions is 12% in an adult population from an endemic area in the archipelago of Turku, in southwestern Finland (Dr. I. Seppälä, University of Helsinki, Helsinki, Finland, personal communication), as against only 2.9% in the adult population in central Finland and in children of the Helsinki area (Dr. S. Kontiainen, Aurora Hospital, Finland, personal communication).

The isolation of *B. burgdorferi* from the blood or cerebrospinal fluid in borreliosis patients has often been unsuccessful. However, the spirochete can be cultivated from most ECM lesions [17,18] although the method is slow and complicated. Direct demonstration of *B. burgdorferi* from the tissue has been performed with a nonspecific silver staining method, and with monoclonal antibodies specific for the outer surface protein A (OspA) of *B. burgdorferi* type strain B31. With such methods, *B. burgdorferi* has also been demonstrated in some cases of morphea [19,20].

The use of polymerase chain reaction (PCR) for the amplification of scarce amounts of microbe-specific DNA from tissue or body fluid samples offers a sensitive and specific tool for diagnostic purposes. Using the PCR technique and subsequent hybridization, as

Manuscript received May 5, 1993; accepted for publication October 27, 1993.

Reprint requests to: Dr. A. Ranki, Department of Dermatology, Helsinki University Central Hospital, Meilahdentie 2, FIN-00250, Helsinki, Finland.

Abbreviations: ACA, acrodermatitis chronica atrophicans; ECM, erythema chronicum migrans; OspA, outer surface protein A.

0022-202X/94/$07.00

little as 0.05 pg of purified *B. burgdorferi* DNA, corresponding to about 25 spirochetes, can be detected [21,22]. As the plasmid-encoded OspA gene is more specific for *B. burgdorferi* than the genus-specific genomic flagellar gene [22–24], we generated oligonucleotide primers and probes specific for the ospA gene to look for *B. burgdorferi* DNA in skin biopsies of patients with ECM, morphea, lymphocytoma, and other dermatoses.

The PCR and hybridization method enabled the demonstration of *B. burgdorferi*–specific DNA in fresh skin biopsies of ECM and lymphocytoma lesions even when specific *Borrelia* antibodies were not present. We also show that Finnish *B. burgdorferi* OspA sequences differ from those of other European strains and from the type strain B31. With primers and probes specific for the Finnish sequences, we were unable to demonstrate *B. burgdorferi*–specific DNA in any of the seven morphea lesions.

MATERIALS AND METHODS

Patients and Tissue Samples Skin biopsies were obtained from 29 patients; nine patients presented with skin lesions clinically suspected to be caused by *B. burgdorferi* infection, seven patients had freshly diagnosed circumscribed scleroderma lesions, five patients showed variable skin lesions and elevated antibody titers to *Borrelia* flagellin antigen, and eight patients had other dermatoses. The skin biopsy was always obtained from the active indurated or erythematous skin area and a neighboring biopsy was obtained for histopathologic examination. The demographic data on the patients are given in Tables 2–4.

Antibodies to *B. burgdorferi* Antibodies to *B. burgdorferi* flagellar protein were routinely determined using the enzyme-linked immunosorbent assay (ELISA) method [3]. Primarily, IgG class antibodies were measured but if elevated titers were obtained, IgM antibodies were also measured. In this assay, antibody titer 500–1000 is regarded as possibly indicative of *B. burgdorferi* infection, and titers above 1000 are considered positive. During this study, the anti-flagellar antibody assay was routinely used in the hospital setting instead of previous less specific antibody assays based on whole cell extracts.

Isolation of *B. burgdorferi* from ECM Skin Lesion DNA extracted from a Finnish *B. burgdorferi* isolate, designated KS1, was used as a positive reference in the PCR assays. The isolate originated from a skin biopsy of a typical ECM lesion on a leg. The spirochete was cultivated in Barbour-Stoenner-Kelly medium [18] modified by substituting fetal bovine serum for rabbit serum and omitting antibiotics for 4 weeks. The isolate was electron microscopically inspected by Dr. Kari Hovind-Hougen at the National Veterinary Laboratory, Denmark, and was morphologically similar to those isolated from Swedish ECM and ACA patients [25].

For DNA isolation, the organisms were pelleted by centrifugation at 10,000 × *g* for 30 min at 37°C, washed (phosphate-buffered saline, 5 mM $MgCl_2$), lysed with non-ionic detergents (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween-20) and digested with proteinase K (0.06 mg/ml, Sigma) at 58°C for 1 h.

Extraction of DNA from Skin Biopsies The DNA for PCR amplification was prepared from frozen tissue samples using a non-ionic detergent method described previously [26]. In addition, DNA was extracted from some samples by the conventional phenol-chloroform extraction method [27]. We found no difference between the two methods in the yield or quality of DNA.

Primers and Probes for PCR Amplification Oligonucleotide primers were synthesized with Millipore Cyclone-DNA-synthesizer using beta amidite chemistry. Three oligonucleotides (OspA 2, OspA 3, and OspA 4) were derived from previously published *B. burgdorferi* type strain B31 OspA sequences ([26], Table I). One additional oligonucleotide OspA 7 was synthesized locating between OspA 3 and OspA 4 (845–863, Genebank accession number X14407). OspA 2 and OspA 4 or OspA 3 and OspA 4 were used as primer pairs. OspA 7 was used as a probe for the latter primer pair. Additionally, another set of OspA-specific primers, SN1 and SN2, and a corresponding probe SN3 (Fig 1) were synthesized, all based on the type strain B31 sequences [22]. This set has been shown to be specific for North American isolates [22]. The SN amplicon obtained from the Finnish *B. burgdorferi* isolate was cloned and sequenced, and the more specific primers BB1 (332–354) and BB2 (405–429), and an oligonucleotide probe, BB3 (360–380, Genbank accession number X14407), were synthesized. To control the quality of the DNA extracted from the skin biopsies, each sample was also amplified with primers specific for human β-globin gene [28]. These amplicons were visualized by ethidium bromide staining of agarose gels (2% NuSieve agarose and 1% SeaKem agarose; FMC BioProducts, Rockland, ME). By amplifying for the β-globin gene under same conditions as for OspA, we also controlled that no inhibitors of amplification were present.

PCR-Based Amplification of *B. burgdorferi*–Specific DNA and Liquid Hybridization to ^{32}P-Labeled Probe Amplification was carried out in 50 ml volume in an automated thermal cycler (Techne PHC-2, Cambridge, UK) in Taq DNA polymerase buffer containing 2.5 U Taq DNA polymerase (Gibco BRL, Gaithesburg, MD), 200 mM dNTPs, 1 mM primers, and 1.5 mM $MgCl_2$. The optimal amplification conditions for each primer pair were determined by adjusting the $MgCl_2$ and primer concentrations and cycling temperatures. For OspA primers, best results, using DNA extracted from the Finnish KS1 *B. burgdorferi* isolate as a template, were obtained with the following amplification temperatures: 94°C 1′ 30″, 50°C 1′ 30″, 72°C 2′, repeated for 30 cycles. PCR amplification with SN1-SN2 primer pair was performed successfully only under nonstringent conditions (94°C 30″, 37°C 30″, and 72°C 1′) for 40 cycles. For the primer pair BB1-BB2, specific for the Finnish KS1 isolate, cycles of 94°C 1′, 60°C 30″, and 72°C 30″ repeated 25 times, were optimal. The optimal $MgCl_2$ concentration for the BB1 + BB2 primer pair was 2 mM. The amplicon was subjected to liquid hybridization to ^{32}P-labeled specific oligonucleotide probe (1.25 pmol, 5 × 10^5 dpm/ml) at 55°C for 30 min. For the OspA and SN probes, hybridization was also attempted at 45°C. The hybridized product was electrophoresed through 10% polyacrylamide gel, and autoradiographed.

The PCR reactions were carried out in a laboratory specifically designed for PCR work to avoid carry-over contaminations. No *Borrelia* plasmids have ever been handled in this space, and the positive control samples were always pipetted last and in a different room with separate air conditioning. Positive-displacement pipettes were used, and the PCR reagents were stored in small aliquots and always handled in a separate room from the patient samples or amplified products.

DNA Sequencing After 3% agarose gel electrophoresis of *B. burgdorferi* DNA amplified with SN1-SN2 primers, a piece of agarose gel containing DNA fragments of the expected length (145 bp [33]) was isolated. The DNA was purified with Sephaglas BandPrep Kit (Pharmacia, Sweden), treated with Klenow enzyme and ligated into Hinc II cleaved M13mp18 vector [27]. After transformation, the recombinants were purified and sequenced according to the Sequenase Version 2.0 protocol (United States Biochemicals, Cleveland, OH). The *B. burgdorferi* sequences were analyzed for maximum homology with BESTFIT program (GCG sequencing analysis software package [29]), and no gaps were allowed. The final alignment was made with LINEUP program [29]. The OspA sequences of the following *B. burgdorferi* isolates were compared to the Finnish sequence: the North-American type strain B31, isolated from an *I. dammini* tick in New York state (Genbank accession number X14407), East-Russian (Siberian) strain Ip90 form *I. persulcatus* tick [37], Swedish ACA patient skin isolate ACAI [17], and the European strains ZQ1 (tick isolate from Germany; accession number X66065), Goe 2 (X60300), Pko skin isolate (X62161), and B29 isolate from *I. ricinus* tick in Germany (M88764).

RESULTS

Identification of OspA Sequences Specific for Finnish *B. burgdorferi* We first tried PCR amplification of the KS1 isolate and some patient samples with OspA 2, 3, and 4 primers. One pg of *B. burgdorferi* DNA was successfully amplified (annealing temperature 50°C or below) with the OspA 3 and OspA 4 primer pair. The obtained amplicon of the expected size of 119 bp in agarose gel electrophoresis did not yield detectable signal on the autoradiographs when hybridized to either labeled OspA3 or OspA7 probe. The same samples were therefore also amplified with another primer pair, SN1 and SN2. With this primer pair, we always obtained an amplified DNA of the correct size (145 bp) both from the cultured isolate of *B. burgdorferi* and from some patient samples. However, formation of nonspecific amplification products was common. We did not manage to eliminate these, even with "hot start" PCR or elevated annealing temperatures. No hybridization to the SN3 probe was obtained, even at low stringency. We therefore cloned the PCR amplification product obtained with the SN1 + SN2 primer pair into M13mp18 vector and sequenced it.

The 98 nucleotide sequence of the Finnish *B. burgdorferi* isolate KS1 (Fig 1) differed in 11 nucleotides (11%) from the North American B31 type strain, and in 13 to 14 nucleotides (13–14%) from published European *B. burgdorferi* sequences [23] but in only five

Table I. *B. burgdorferi*-Specific Oligonucleotide Primers and Probes Used[a]

Primers	Probe	Reference	Finnish *B. burgdorferi* Isolate	
			Amplification	Hybridization
OspA2: 889–1003 OspA3: 876–851	OspA4: 758–783	Persing DH, *et al,* 1990 [34]	+, weak	–
OspA4: 758–783 OspA3: 876–851	OspA7: 815–833		+	–
SN1: 306–331 SN2: 450–427	SN3: 360–394	Nielsen SL, *et al,* 1990 [33]	++	–
BB1: 332–354 BB2: 405–429	BB3: 360–386	Ranki A, *et al,* 1993	+++	+++

[a] Nucleotide locations refer to *B. burgdorferi* type strain B31 (Genbank accession number X14407) OspA sequence.

nucleotides from a German skin isolate [30] and in 1 nucleotide from the Swedish ACA-1 isolate [31]. In the region of the SN3 probe, there was a mismatch of seven nucleotides (20%) in the Finnish strain compared with the American type strain B31. We therefore synthesized a new set of primers (BB1 and BB2, 332–354 and 429–405, Genbank accession number X16467). Using these primers at annealing temperatures of 55–60°C in 25 PCR cycles, amplified DNA (98 bp) that hybridized to the internal probe BB3, was obtained from the Finnish ECM isolate KS1 (Fig 2, Table I). A significantly weaker signal was obtained when either SN1 or SN2 was used as the other primer (product of 119 bp in Fig 2). Under the same conditions, no hybridizing product was obtained with the type strain-specific SN1 + SN2 primer pair alone (Fig 2).

Amplification and Hybridization Results in Clinical Samples Altogether eight patients presenting with annular or large red, indurated skin lesions were clinically suspected of ECM. The diagnoses were confirmed histologically. The time between possible or documented tick bite and the biopsy varied from 2 weeks to about 6 months. One patient (number 6, Table II) had received cefalexine prior to the biopsy. Using the oligonucleotides specific for Finnish *B. burgdorferi* strain, specific DNA was successfully amplified and hybridized from the skin biopsies of six of the seven untreated ECM patients (Table II and Fig 3). Interestingly, only two of the patients had detectable antibodies (at suspicious level) to *Borrelia* flagellin antigen at the time of the biopsy for PCR (Table II), and a third patient (number 8) had IgM class antibodies only. In a further patient, a rise in the antibody titer was observed only after treatment with amoxicillin.

The PCR-based hybridization was negative in one ECM patient treated a month earlier and in another patient with a large ECM on the abdomen and thighs (Table II). It is possible that in the latter case the biopsy was not obtained from an area with spirochetes. A third patient, who presented with an erythematous ECM-suspect lesion on the arm and who had spent the summer in Austrian alps, was also PCR negative (Fig 3). He might have been infected with a non-homologous *B. burgdorferi* strain.

In this series of patients, a 3-year-old boy presented with a scrotal lymphocytoma (Table II). The biopsy of this lesion gave a strong positive PCR-based hybridization signal for *B. burgdorferi.* The boy was subsequently treated with amoxicillin, and the lesion resolved completely.

The specificity of the BB1, BB2, and BB3 sequences was confirmed when no *B. burgdorferi*-specific DNA was detected in eight other patients with skin lesions not typical of or not suggestive of borreliosis (Table III). None of these patients had raised antibody titers to flagellin. Histopathology revealed various other dermatoses in all these patients.

Skin biopsies from six patients with high antibodies to *B. burgdorferi* but with diverse skin manifestations were also studied (Table IV). One patient presented with localized scleroderma. She received intravenous ceftriaxone treatment (2 g/d for 14 d), but there was no change in the antibody titer 4 months later. Three other patients had received extensive previous antibiotic therapy, either with oral penicillin or with intramuscular ceftriaxone, for a suspected borreliosis. None of the patients had anti-cardiolipin antibodies. At the time of the biopsy for the PCR analysis, the histopathologic analysis did not reveal any changes suggestive of ECM or ACA in any of the patients. All these patients were negative in the PCR analysis with the Finnish primers and probe. Despite this, all patients received antibiotic treatment (ceftriaxone intravenously for 14 d, amoxicillin for 3 months, or doxycycline for 1 to 3 months), but no decrease in antibody titers was seen in three of four patients during follow-up (Table IV).

When skin biopsies of the active borders of altogether seven patients with histologically confirmed morphea (Tables III and IV) were analyzed with the PCR method using either oligonucleotides based on the Finnish ospA sequence or those based on the type-strain sequence, no hybridization signal was obtained (Fig 4). The results were negative even when low stringency or repeated analyses were performed.

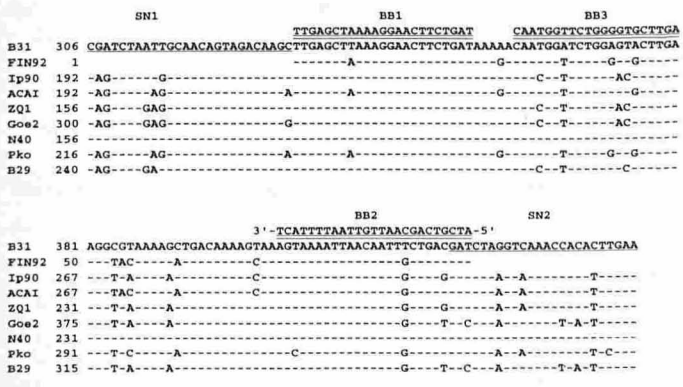

Figure 1. Nucleotide sequence (FIN) of the ospA gene region 332–429 of Finnish *B. burgdorferi* isolate KS1 in comparison to North-American (B31 type strain), East-Russian (Ip90), Scandinavian (ACAI), and Middle-European (ZQ1, Goe2, Pko, B29) strains. The primers (BB1 and BB2) and probe (BB3) based on the Finnish sequence are shown.

DISCUSSION

We demonstrate that the Finnish *B. burgdorferi* strain, isolated from an ECM skin lesion, shows genetic heterogeneity in its OspA in comparison with *B. burgdorferi* isolates from other geographic areas. Due to this heterogeneity, the demonstration of *B. burgdorferi*-specific DNA of skin biopsies from other Finnish patients was successful only after the partial sequence of the ospA gene in the native *B. burgdorferi* was identified.

There has been a need for the accurate demonstration of *B. burgdorferi* spirochete in early infections, in which an antibody response to the flagellar antigen is detected only in 36–70% of cases [3]. As borrelial flagella resemble those of other treponemes and other gram-positive bacteria [25], the specificity of the antibody test has also been a problem sometimes. Also, it has become evident that not

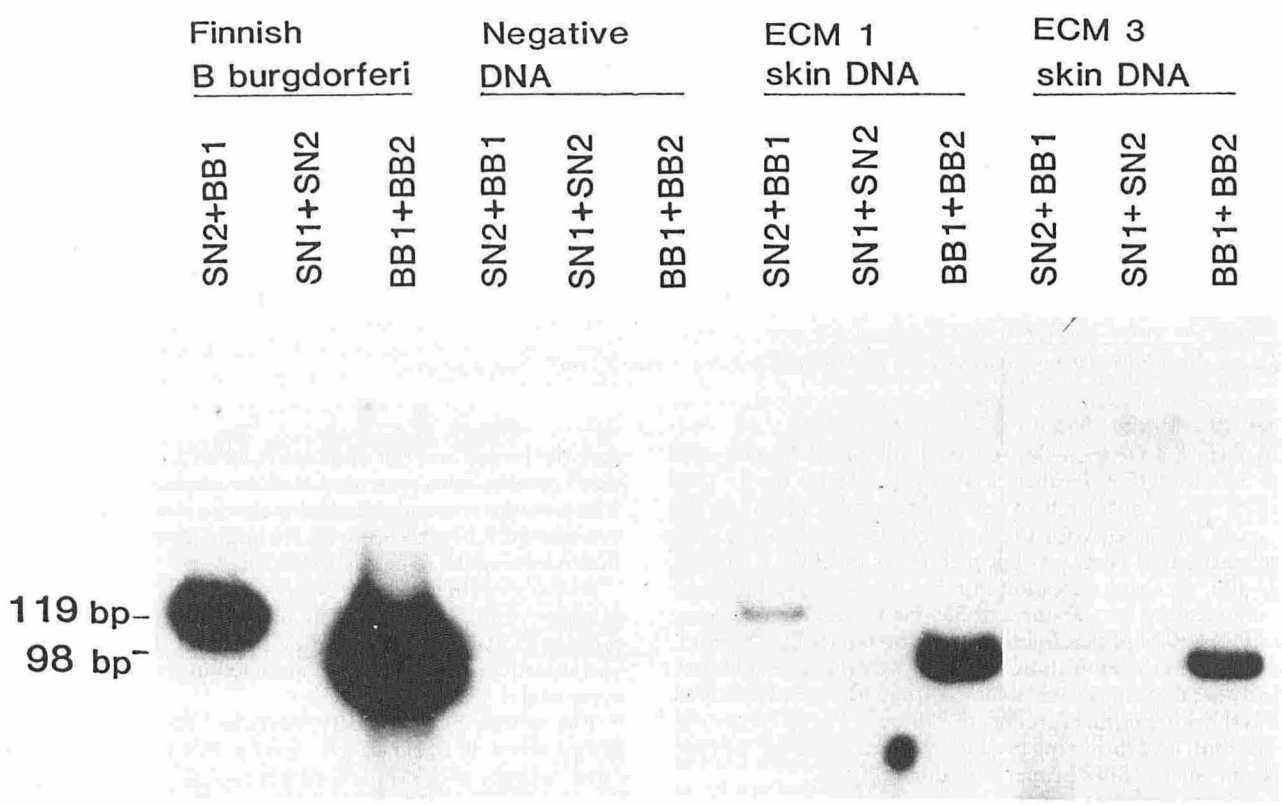

Figure 2. Hybridization of a Finnish ECM *B. burgdorferi* isolate and of ECM skin lesion-derived DNA samples to ^{32}P-labeled oligonucleotide probe (BB3), based on the Finnish OspA sequence, after PCR amplification with primers based on North-American type strain (SN1, SN2) or the Finnish OspA (BB1, BB2) nucleotide sequences. ECM1 and ECM3 denote patients in Table II.

only erythema chronicum migrans but also some atypical erythematous skin lesions, as well as benign cutaneous lymphadenosis, morphea-like skin lesions, and even lichen sclerosus et atrophicus, may be associated with borreliosis [7–9].

PCR amplification of the plasmid-encoded OspA gene has been previously shown to be highly specific for *B. burgdorferi* [24], as the linear plasmid coding for OspA is not found in other procaryotic organisms [32]. The OspA protein, which is exposed on the surface of the microbe unlike flagellin, is of special interest as it is associated with the virulence of the spirochete [32,33], and, also, as it is the major target of a protective immune response [34–36]. Using primers and corresponding probes first from the C-terminal part of

Table II. Detection of *B. burgdorferi* OspA-Specific DNA by PCR Amplification and Hybridization in Comparison with Serologic Findings

Clinical Diagnosis	PCR-Based Hybridization Result[a]	Anti-Flagellar Antibodies (time after PCR)	Treatment History[b]
1. ECM	+	Negative[c] Negative (2 months)	Tick bite 3 weeks earlier; doxycycline 14 d → cured
2. ECM	+	Suspicious Negative (up to 5 months)	Amoxicillin 2 weeks → cured
3. ECM	+	Negative Positive (1 month) Negative (5 months)	Tick bite 5 months earlier; amoxicillin 2 weeks → cured
4. ECM	+	Positive Suspicious (3 weeks) Positive (5 months)	ECM in buttock for 2 months; amoxicillin 1 month → cured
5. ECM	–	Negative Negative (1 month)	ECM in lower abdomen for 1.5 months; penicillin 3 weeks → cured
6. ECM, status post	–	Suspicious Negative (up to 3 months)	ECM 1 month earlier: cefalexine 10 d; doxycycline 10 d → cured
7. ECM	+	Negative	Tick bite in Estonia 2–3 weeks earlier
8. ECM	+	Suspicious	Tick bite 2–3 weeks earlier
9. Lymphocytoma	+	Suspicious	Lesion for 2 months in scrotum, amoxicillin 3 weeks → cured

[a] Oligonucleotides specific for the Finnish strain of *B. burgdorferi* were used.
[b] Dosages used: doxycycline 200 mg/d orally, amoxicillin 2 g/d orally (children 40 mg/kg), ceftriaxone 2 g/d intravenously.
[c] Negative indicates ELISA titer below 500, suspicious = titer 500–1000, and positive = titer above 1000.

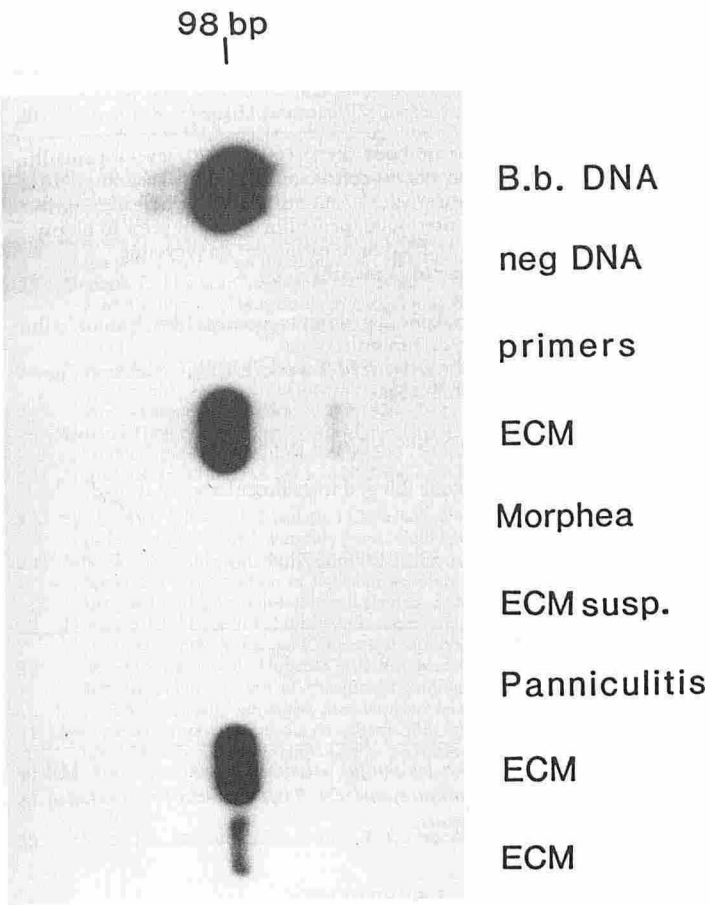

Figure 3. PCR amplification and hybridization results of skin DNA samples from three ECM patients, one patient suspected for ECM, one with morphea, and one with panniculitis. The Finnish ECM isolate KS-1 (designated B.b. DNA) served as a positive control.

the ospA sequence and then from the N-terminal part, we obtained amplified DNA of the correct size from DNA extracted from a Finnish *B. burgdorferi* isolate, and estimated that the detection limit was 0.01 pg. A similar quantity of HIV DNA was amplified in parallel experiments, and other groups have been able to amplify the same amount of target DNA with *B. burgdorferi*-specific chromosomal primers [21]. However, we were unable to hybridize the amplified DNA, even under low-stringency conditions to probes deduced from the type strain B31 sequence. We therefore cloned and sequenced the amplified product.

The 98 nucleotide sequence of the Finnish ECM isolate, KS1, OspA gene (FIN) showed equal nucleotide divergence (11 to 14%) from the North American type strain (B31), from German *I. ricinus* tick isolates (ZQ1, B29) and from an East Russian (Siberian) *I. persulcatus*-tick isolate (Ip90) [31,37]. The FIN sequence showed most (six of 30, 20%) diversity in the region for which the type strain-based oligonucleotide probes were designed and this would explain the negative hybridization results. It is of interest to note that there was only one to five nucleotide differences in FIN compared with sequences available from two other borreliosis skin isolates, namely, the geographically close Swedish acrodermatitis chronica atrophicans isolate ACA-1 and a German skin isolate Pko [17,30]. The two latter isolates fall into the same branch in the phylogenetic tree, based on amino acid homologies [23], and they are most distant from the North American strains. Thus, it is possible that geographical difference in the clinical syndromes of Lyme borreliosis would be reflected in genomic variability of the OspA, as in Europe ECM lymphocytoma and ACA are the prevalent symptoms and in the United States arthritis is more common.

Using the new set of oligonucleotides based on our native ospA sequence, we obtained a strongly positive PCR result, confirmed by hybridization to a specific probe, in six of the seven untreated ECM lesions and in the one lymphocytoma lesion. It is noteworthy that only two of these patients had antibodies to *B. burgdorferi* flagellin at the time of the biopsy. However, one additional ECM patient, treated with cefalexine 1 month earlier, gave a negative PCR result.

Our results thus extend the recent findings of Melchers and co-workers [38] and Schwarz and co-workers [39]. The first group found *B. burgdorferi*-specific DNA after application of nested primers specific for the genomic DNA, in three of four skin biopsies from ECM patients and in most ACA lesions, but not in a morphea lesion. However, the use of a single set of primers, like in our method, instead of nested PCR is always much safer in a clinical diagnostic setting when carry-over contamination of the samples must be avoided. Schwartz and co-workers [39], using primers and probes specific for the 23S rRNA sequences, achieved a sensitivity of 59 to 62% in demonstrating *B. burgdorferi* in 35 untreated ECM skin lesions. However, their PCR method was positive in only nine of 19 seronegative ECM patients although *B. burgdorferi* isolates from three geographically distant areas were positive with the assay. Our Finnish isolates have not yet been tested with these 23S rRNA primers.

All the samples of ours other than the ECM patients, including those with morphea, gave a repeatedly negative PCR result. As the morphea samples were also negative with the type strain-specific primers, even under low stringency, it is unlikely that a *B. burgdorferi* strain different from the geographically typical strain was present in the morphea lesions. We were thus unable to confirm previous findings, based on serology, immunohistochemistry, or nested PCR with genus-specific flagellar primers, of an association between *B. burgdorferi* and morphea [10,11,40,41]. It may, however, be possible that morphea is caused by another *B. burgdorferi* strain with either a divergent or a completely missing ospA gene.

The group of patients with persistently elevated antibody titers to *Borrelia* flagellin and with various skin lesions were perhaps the most cumbersome in terms of clinical decision making. In all these patients, most of whom had previously received antibiotic treatment for a suspected borreliosis, the PCR results were completely negative. The possible reasons for the negative result, despite sustained anti-flagellin antibody response, are that in some patients a prolonged serological scar might survive after a borrelial infection, or that the number of spirochetes in the lesions was below the detection limit of our method, or that a strain with considerably divergent ospA gene was the causative agent. Our findings would favor the first possibility as the patients did not develop any symptoms typical of borreliosis during a follow-up of up to 2 years.

In conclusion, we have shown that a PCR method, applied directly to skin biopsy samples, is a reliable diagnostic tool for early borreliosis even when a specific antibody response is lacking. We also show that the ospA gene, coded by a linear plasmid typical for *B. burgdorferi*, found in Finnish *B. burgdorferi* skin isolates differs

Table III. Detection of *B. burgdorferi*-specific DNA by PCR Amplification and Hybridization in Skin Biopsies of Circumscribed Scleroderma (morphea) and in Other Dermatoses in Relation to Serologic Findings

Patient Group	Number of Patients	PCR-Based Hybridization Results[a]	Anti-Flagellar Antibodies[b]
Morphea	6	Negative	<200–580
Other dermatoses[c]	5	Negative	<200
Condylomata acuminata	3	Negative	Not determined

[a] Oligonucleotides BB1, BB, and BB3 specific for the Finnish *B. burgdorferi* were used.
[b] Based on repeated measures.
[c] Includes eczema, lupus erythematosus, melanosis, plasma cell infiltrate.

Table IV. Results of PCR Amplification and Hybridization of *B. burgdorferi* OspA-Specific DNA in Skin Biopsies of Patients with Persistently Elevated Antibodies to *B. burgdorferi* Flagellin in Relation to Therapeutic Efforts

Patient	Clinical Diagnosis	PCR-based Hybridization[a]	Anti-Flagellar Antibodies (follow-up time in months)	Treatment History
KH	Morphea	−	710 1000 (4)	Previous antibody titers: 1600–2700, several penicillin treatments; → ceftriaxone 2 g/d intravenously, 14 d
GE	Panniculitis	−	1200 2600 (2)	Borreliosis treated 7 months earlier, whereafter antibody titer 4000, penicillin 1 month prior to biopsy
ES	Hyperpigmented macules	−	3400 3300 (1) 5600 (4) 3800 (6) 6100 (10)	Penicillin 2.5 month earlier, → doxycycline 200 mg/d, 3 months → ceftriaxone 2 g/d intravenously, 14 d + amoxicillin 1.5 g/d, 3 months
MR	SCLE[b], suspected		2060 560 (1.5) 880 (10)	Prednisone 40 mg/d 2 weeks earlier, → tetracycline 1 g/d, 3 weeks
RB	LSA[b]	—	2000 Lost to follow-up	→ tetracycline 1 g/d, 3 weeks
VK	Anetoderma	—	8710 7100 (2) 4400 (5) 7100 (11) 2400 (16) 270 (20)	Ceftriaxone 2.5 g/d intramuscularly, 12 d → doxycycline 200 mg/d, 1 month

[a] Oligonucleotides specific for the Finnish type of *B. burgdorferi* were used.
[b] SCLE, subacute cutaneous lupus erythematosus; LSA, lichen sclerosus et atrophicus.

from that of other European and North American isolates. The genetic heterogeneity of *B. burgdorferi* isolates has been recently disclosed [4,30,42–44]. The isolates can be grouped into six different ospA genotypes, five of which are found among European isolates, whereas American isolates almost exclusively fall into one group only and show sequence homologies of >99% [23]. Most amino acid divergence has been described in the middle region and in the C-terminal half of OspA [36,44], but the Finnish sequence clearly covers a more N-terminal region. It is noteworthy that we were able to demonstrate *B. burgdorferi*–specific DNA with oligonucleotides specific for the Finnish strain also in a skin lesion resulting from a tick bite in Estonia, and the Finnish ospA sequence was almost identical to that of the Swedish ACA isolate. Thus, it seems that the *B. burgdorferi* isolates around the Baltic sea are closely related.

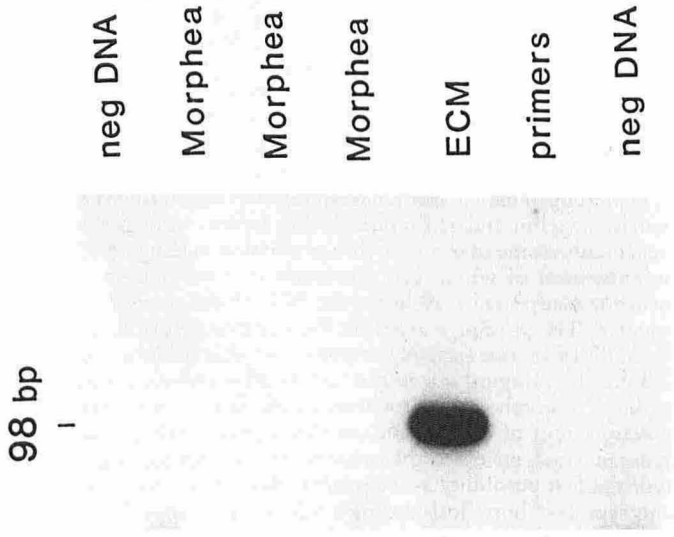

Figure 4. *B. burgdorferi* ospA-specific PCR amplification and hybridization results of morphea skin samples with oligonucleotides specific for the Finnish strain.

We thank Ms. Kaija Järvinen for skillful technical assistance, and Dr. Håkan Granlund, Dr. Synnöve Mynttinen, and Dr. Timo Reunala for cooperation in obtaining some of the skin biopsies.

REFERENCES

1. Grodzicki RL, Steere AC: Comparison of immunoblotting and indirect enzyme-linked immunosorbent assay using different antigen preparations for diagnosing early Lyme disease. *J Infect Dis* 157:790–797, 1988
2. Magnarelli LA, Anderson JF, Barbour AG: Enzyme-linked immunosorbent assays for Lyme disease: reacting to subunits of *B. burgdorferi*. *J Infect Dis* 159:43–49, 1989
3. Hansen K, Åsbrink E: Serodiagnosis of erythema migrans and acrodermatitis chronica atrophicans by the *Borrelia burgdorferi* flagellum enzyme-linked immunosorbent assay. *J Clin Microbiol* 27:545–551, 1989
4. Stanek G, Wewalka G, Groh V, Neumann R, Kristoferitsch W: Differences between Lyme disease and European arthropod-borne Borrelia infections (letter). *Lancet* I:401, 1985
5. Shrestha BS, Grodzicki MS, Steere AC: Diagnosing early Lyme disease. *Am J Med* 78:235–240, 1985
6. Steere AC: Medical progress: Lyme disease. *N Engl J Med* 321:586–596, 1989
7. Abele DC, Kenya HA: The many faces and phases of borreliosis I. Lyme disease. *J Am Acad Dermatol* 23:167–186, 1990
8. Åsbrink E, Hovmark A: Lyme Borreliosis: aspects of tick-borne *Borrelia burgdorferi* infection from a dermatologic viewpoint. Semin Dermatol 9:277–291, 1990
9. Malane MS, Grant-Kels JM, Feder HM, Luger SW: Diagnosis of Lyme disease based on dermatologic manifestations. *Ann Int Med* 114:490–498, 1991
10. Aberer E, Neumann R, Stanek G: Is localized scleroderma a borrelia infection? *Lancet* II:278, 1985
11. Aberer E, Klade H, Stanek G, Gebhart W: *Borrelia burgdorferi* and different types of morphea. *Dermatologica* 182:145–154, 1991
12. Hoesly J, Mertz L, Winkelmann KK: Localized scleroderma (morphea) and antibody to *Borrelia burgdorferi*. *J Am Acad Dermatol* 17:455–458, 1987
13. Halkier-Sörensen L, Kragballe K, Hansen K: Antibodies to the *Borrelia burgdorferi* flagellum in patients with scleroderma, granuloma annulare and porphyria cutanea tarda. *Acta Derm Venerol* 69:116–119, 1989
14. Raguin G, Boisnic S, Soyteyrand P, Baranton G, Piette JC, Godeau P, Frances C: No evidence for a spirochaetal origin of localized scleroderma. *Br J Dermatol* 127:218–220, 1992
15. Fahrer H, vd Linden S, Sauvain MJ, Gern L, Zhioua E, Aeschlimann A: The prevalence and incidence of clinical and asymptomatic Lyme borreliosis in a population at risk. *J Infect Dis* 163:305–10, 1991
16. Huycke M, D'Alessio D, Marx J: Prevalence of antibody to *Borrelia burgdorferi* by indirect fluorescent antibody assay, ELISA, and western immunoblot in healthy adults in Wisconsin and Arizona. *J Inf Dis* 165:1133–1137, 1992
17. Åsbrink E, Hovmark A: Successful cultivation of spirochetes from skin lesions of patients with erythema chronicum migrans Afzelius and acrodermatitis chronica atrophicans. *Acta Pathol Microbiol Immunol Scand B* 93:161–163, 1985
18. Berger BW, Kaplan MH, Rothenberg IR, Barbour AG: Isolation and character-

ization of the Lyme disease spirochete from the skin of patients with Erythema chronicum migrans. *J Am Acad Dermatol* 13:444–449, 1985
19. Aberer E, Stanek G, Ertl M, Neumann R: Evidence for spirochetal origin of cirumscribed scleroderma (morphea). *Acta Derm Venerol* 67:225–231, 1987
20. Scott AR, Sanchez JL, Taboas JO: Spirochetal forms in the dermal lesions of morphea and lichen sclerosus et atrophicus. *Am J Dermatopathol* 12:357–362, 1990
21. Rosa PA, Schwan TG: A specific and sensitive assay for the Lyme disease spirochete *Borrelia burgdorferi* using the polymerase chain reaction. *J Inf Dis* 160:1018–1029, 1989
22. Nielsen SL, Young KKY, Barbour AG: Detection of *Borrelia burgdorferi* DNA by the polymerase chain reaction. *Mol Cell Probes* 4:73–79, 1990
23. Wallich R, Helmes C, Schaible UE, Lobet Y, Moter SE, Kramer MD, Simon MM: Evaluation of genetic divergence among *Borrelia burgdorferi* isolates by use of OspA, fla, HSP60, and HSP70 gene probes. *Infec Immun* 60:4856–4866, 1992
24. Persing DH, Telford III SM, Rys PN, Dodge DE, White TJ, Malawista SE, Spielman A: Detection of *Borrelia burgdorferi* DNA in museum specimens of Ixodes dammini ticks. *Science* 249:1420–1423, 1990
25. Hovind-Hougen K: Ultrastructure of spirochetes isolated from Ixodes ricinus and Ixodes dammini. *Yale J Biol Med* 57:543–548, 1984
26. Higuchi R: In: Erlich HA (ed.). *PCR Technology*. Stockton Press, Stockton, p 31, 1989
27. Sambrook J, Fritsch EF, Maniatis T: *Molecular cloning: a laboratory manual, 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989
28. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Elrich HA, Arnheim N: Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354, 1985
29. Devereux JR, Haeberli P, Smithies O: A comprehensive set of sequence analysis programs for the VAX and Convex systems. *Nucl Acad Res* 12:387–395, 1984
30. Zumstein G, Fuchs R, Hoffman A, Preac-Mursic V, Soutschek E, Wilske B: Genetic polymorphism of the gene encoding the outer surface protein A (OspA) of *Borrelia burgdorferi*. *Med Microbiol Immunol* 181:57–70, 1992
31. Jonsson M, Noppa L, Barbour AG, Bergström S: Heterogeneity of outer membrane proteins in *Borrelia burgdorferi*: comparison of osp operons of three isolates of different geographic origins. *Infect Immun* 5:1845–1853, 1992
32. Barbour A, Hayes S: Biology of *Borrelia burgdorferi* species. *Microbiol Rev* 50:381–400, 1986
33. Schwan TG, Burgdorfer W, Garon CF: Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. *Infect Immun* 56:1831–1836, 1988
34. Fikrig E, Barthold SW, Kantor FS, Flavell RA: Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. *Science* 250:553–556, 1990
35. Simon MM, Schaible UE, Kramer MD, Eckerskorn C, Museteanu C, Müller-Hermelink HK, Wallich R: Recombinant outer surface protein A from *Borrelia burgdorferi* induces antibodies protective against spirochetal infection in mice. *J Infect Dis* 164:123–132, 1991
36. Fikrig E, Barthold SW, Persing DH, Sun X, Kantor FS, Flavell RA: *Borrelia burgdorferi* strain 25015: characterization of outer surface protein A and vaccination against infection. *J Immunol* 148:2256–2260, 1992
37. Kryuchechnikov VN, Korenberg EI, Scherabkov SV, Yovalevsky YV, Levin ML: Identification of Borrelia isolated in the USSR from *Ixodes persulcatus* ticks. *J Microbiol Epidemiol Immunobiol* 12:41–44, 1988
38. Melchers W, Meis J, Rosa P, Glaas E, Nohlmans L, Koopman R, Horrevorts A, Galama J: Amplification of *Borrelia burgdorferi* DNA in skin biopsies from patients with Lyme disease. *Clin Microbiol* 29:2401–2406, 1991
39. Schwartz I, Wormser GP, Schwartz JJ, Cooper D, Weissensee P, Gazumyan A, Zimmermann E, Goldberg NS, Bittker S, Campbell GL, Pavia CS: Diagnosis of early Lyme disease by polymerase chain reaction amplification and culture of skin biopsies from erythema migrans lesions. *J Clin Microbiol* 30:3082–3088, 1992
40. Weber K, Preac-Mursic V, Reimers CD: Spirochetes isolated from two patients with morphea. *Infection* 16:29–30, 1988
41. Schempp C, Bocklage H, Lange R, Kölmer HW, Orfanos E, Gollnick H: Further evidence for *Borrelia burgdorferi* infection in morphea and lichen sclerosus et atrophicus confirmed by DNA amplification. *J Invest Dermatol* 100:717–720, 1993
42. Barbour AG, Heiland RA, Howe TR: Heterogeneity of major proteins in Lyme disease borreliae: a molecular analysis of North American and European isolates. *J Inf Dis* 152:478–484, 1985
43. LeFebvre RB, Perng GC, Johnson RC: Characterization of *Borrelia burgdorferi*, isolates by restriction endonuclease analysis and DNA hybridization. *J Clin Microbiol* 27:636–639, 1989
44. Wilske B, Luft B, Schubach W, Zumstein G, Jauris S, Preac-Mursic V, Kramer M: Molecular analysis of the outer surface protein (OspA) of *Borrelia burgdorferi* for conserved and variable antibody binding domains. *Med Microbiol Immunol* 181:191–207, 1992