Original Report

Rapid Diagnosis of Lyme Disease: Flagellin Gene–Based Nested Polymerase Chain Reaction for Identification of Causative *Borrelia* Species

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ABSTRACT

Objective: Each of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* has characteristic restriction sites in its flagellin gene. The authors focused on this gene and developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis for rapid diagnosis of Lyme disease.

Methods: External and internal primer sets were designed for nested PCR to amplify an approximately 580 bp fragment of the flagellin gene that includes species-specific restriction sites. DNA extracted from tissue samples of mice and humans were used as templates for PCR. The amplicons obtained were digested with *Hap*II, *Hha*I, *Cel*II, *Hin*cII, or *Dde*I endonuclease.

Results: In mice experimentally infected with each of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, borrelial DNA was detected irrespective of differences in the causative species. However, RFLP of the amplicons was able to identify the species. Skin biopsy samples from 11 Japanese patients with erythema migrans were subjected to both PCR and culture tests. Borrelial infections were detected in seven cases (64%) by PCR and eight cases (73%) by culture. All PCR-positive samples were also positive by culture. The causative species in human infections was easily identified as *B. garinii* by RFLP analysis of the amplicons.

Conclusion: The nested PCR-RFLP system appears to be an easy and reliable diagnostic tool for the detection and species identification of borreliae in human cutaneous biopsies.

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Lyme disease caused by *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, or *Borrelia afzelii* is the most common tick-borne illness in North America and Eurasia.[1,2] Although all three species prevail in Europe, only *B. burgdorferi* sensu stricto is found in North America. In the Far East, both *B. garinii* and *B. afzelii* are found.[3–5] The organisms are transmitted to humans by bites of infected ticks of the *Ixodes ricinus* species complex. Infection in humans leads to a variety of manifestations, including dermatopathy, cardiopathy, neuropathy, and arthropathy.[6] Epidemiologic and clinical data in Europe suggest that each of the Lyme disease pathogens has a characteristic pathogenic potential.[1,2,7,8] Infection with *B. burgdorferi* sensu stricto seems to cause arthritis, whereas neurologic symptoms are associated with *B. garinii*. The late cutaneous symptom of acrodermatitis chronica atrophicans appears to be related to *B. afzelii*. The early cutaneous manifestation of erythema migrans around the sites of tick bites frequently occur irrespective of differences in the causative species. Although erythema migrans and acrodermatitis chronica atrophicans are characteristic for Lyme disease, many symptoms involving internal organs are less characteristic. Therefore, confirmation of the clinical diagnosis is attempted mostly by measuring antibodies against borreliae. However, the diagnostic value of serologic tests is substandard because of low sensitivity and cross-reactivity.[9,10] The results of serologic tests may also be confused by the polymorphism of immunogenic outer surface proteins.[11–14]

Recent phylogenetic studies demonstrated several taxons related to the Lyme disease borreliae.[15–18] At present, *Borrelia andersonii* and group DN127 in North America,[17,18] groups PotiB2 and VS116 in Europe,[18] and *Borrelia japonica*, *Borrelia tanukii*, and *Borrelia turdae* in Japan[19,20] are recognized and have been isolated from ixodid ticks which are able to feed on humans. Moreover, *Borrelia miyamotoi* in Japan and *Borrelia lonestari* in North America, which are related to relapsing fever borreliae, have been found in ixodid ticks.[21,22] The pathogenicity of these different taxons is unresolved, and

reliable methods for identifying the pathogens from clinical samples are needed for diagnostic laboratories. Previous phylogenetic analysis of *Borrelia* species revealed that their flagellin genes have a marked utility in taxonomy.[16] Based on the alignment data of the flagellin gene sequences of various *Borrelia* species, the authors developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis for distinguishing the Lyme disease borreliae from other related taxons.[16] In the current study, this technique was applied to clinical diagnosis of Lyme disease and evaluated its diagnostic value in comparison with the ordinary culture methods for isolating borreliae. For detecting borrelial DNA from tissue samples, the sensitivity of PCR was increased by using a nested method, using external and internal primer sets. The PCR analysis was first performed in tissue samples from mice experimentally infected with *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii* and then applied to cutaneous biopsy samples from erythematous lesions of tick-bite victims in Japan.

MATERIAL AND METHODS

Mice

Female BALB/c mice (7- or 8-weeks-old), which were bred in the Animal Laboratory for Medical Research, Asahikawa Medical College, were used for all experiments. The mice were infected with borreliae by intraperitoneal inoculation of approximately 10^7 cultured cells or by bites of infected nymphal ticks of *Ixodes persulcatus*.

Borreliae

Low-passaged strains 297 and NCH-1 of *B. burgdorferi* sensu stricto were used for infecting mice. These were kindly provided by T. Masuzawa, University of Shizuoka, Japan. Other low-passaged strains used were *B. garinii* N346 and *B. afzelii* N391. These were the original isolates from *I. persulcatus* in Hokkaido, Japan. *Borrelia garinii* JEM6 isolated from an erythema lesion of a patient with Lyme disease in Hokkaido were also used.[23] According to the system defined by Piesman,[24] this strain has been maintained by alternate passage between *I. persulcatus* ticks and Mongolian jirds (*Meriones unguiculatus*). The JEM6-infected nymphs were reared from replete larvae that had fed on infected jirds. Infection of mice with the JEM6 strain was induced by exposure of 12 infected nymphs. The infection rate of nymphs used was approximately 100% as determined by direct-fluorescent antibody technique.[25]

Culture

A BSK II medium containing rifampin (50 μg/mL) was used for isolating borreliae.[26] Minced tissue samples (approximately 10 mg) were individually inoculated into 6 mL of medium in a culture tube. The tubes were incubated at 31°C and were examined for borreliae by dark-field microscopy biweekly for 4 weeks. In cutaneous samples from mice and humans, the skin surface was wetted with 10% povidone-iodine solution and cleaned with 70% ethanol before samples were taken.

Template DNA

A spin column kit (QIAamp Tissue Kit; QIAGEN Inc., Chatsworth, CA) was used for rapid purification of DNA from tissue samples. Using the attached buffer system, DNA was extracted as recommended by the manufacturer. In brief, approximately 10 mg of tissue (3 to 4 mm^3) was incubated in lysis buffer at 55°C for 3 hours, and then DNA was absorbed in a spin column. After the spin column was washed, DNA was eluted with 400 μL elution buffer. The eluted DNA solution was directly used as a template for nested PCR. The concentration of DNA was determined spectrophotometrically by measuring the A_{260} (range, 30 to 60 μg/mL in murine samples; 15 to 30 μg/mL in human samples).

Nested Polymerase Chain Reaction

Oligonucleotide primers were prepared by a custom synthesis service (Funakoshi Co. Ltd., Tokyo, Japan). The external primer set used was as follows: 5′-TCT GAT GAT GCT GCT GGT ATG G-3′ (sense primer A) and 5′-CTG CTA CAA CCT CAT CTG TCA T-3′ (antisense primer B). The previously published set was used as an internal primer set: 5′-GCA GTT CAA TCA GGT AAC GG-3′ (sense primer C) and 5′-AGG TTT TCA ATA GCA TAC TC-3′ (antisense primer D).[16] Those primers were designed from the flagellin gene sequence of *B. garinii* HT22.[3] Primers A and B are located at the nucleotide positions 118 to 139 and 898 to 919, respectively. Primers C and D are located at 280 to 299 and 844 to 863, respectively. Those positions are correspondent to the conserved region of the flagellin genes of Lyme disease borreliae and their relative species. All primers were *Borrelia*-specific, as confirmed by using the basic local alignment search tool (BLAST) algorithm and the GenBank database. The first PCR was carried out in a 25 μL (total volume) reaction mixture containing 2.5 μL template DNA, each deoxynucleotide triphosphate at a concentration of 200 μM, each external primer at a concentration of 0.1 μM, 1U of *Taq* polymerase (Ex *Taq*; Takara Co. Ltd., Kyoto, Japan), and Ex *Taq* reaction buffer. The reaction mixtures were overlaid with mineral oil and were subjected to 30 thermal cycles consisting of 94°C for 40 seconds, 63°C for 60 seconds, and 72° for 60 seconds. After amplications, 2.5 μL of the first PCR was transferred to a second PCR consisting of the same reaction mixture, but instead of external primers each internal primer at a concentration of 0.5 μM was added. The second PCR was run through 30 cycles of 94°C for 40 seconds, 50°C for 40 sec-

onds, and 72°C for 40 seconds. To examine the amplified product, a 5 μL aliquot of the reaction mixture was electrophoresed in 2.5% agarose gel (NuSieve GTG; FMC BioProducts, Rockland, ME) with TBE buffer (100 mM Tris; 90 mM boric acid; 1 mM EDTA, pH 8.3). Amplified fragments were visualized by ethidium bromide staining. DNA ladders, 100 bp ladder (100–1000 bp) and 20 bp ladder (20–1000 bp) (GenSura Laboratories, Del Mar, CA), were used as size markers.

A panel of positive and negative controls was included in each nested PCR analysis to monitor contamination and false negative amplification. Template DNA extracted from earlobe tissues of mice infected with *B. burgdorferi* sensu stricto 297 was used as a positive control and template DNA from earlobes of normal mice was used as a negative control.

Restriction Fragment Length Polymorphism

As shown in Figure 1, the expected PCR product was an approximately 580 bp fragment, including species-specific restriction sites. The amplicons obtained were digested with *Hap*II, *Hha*I, *Hin*cII (Takara), *Cel*II (Pharmacia, Uppsala, Sweden), or *Dde*I (TOYOBO, Osaka, Japan) as recommended by suppliers. The endonuclease digests were electrophoresed and resultant RFLP patterns were classified into flagellin (fla) types as previously reported.[16]

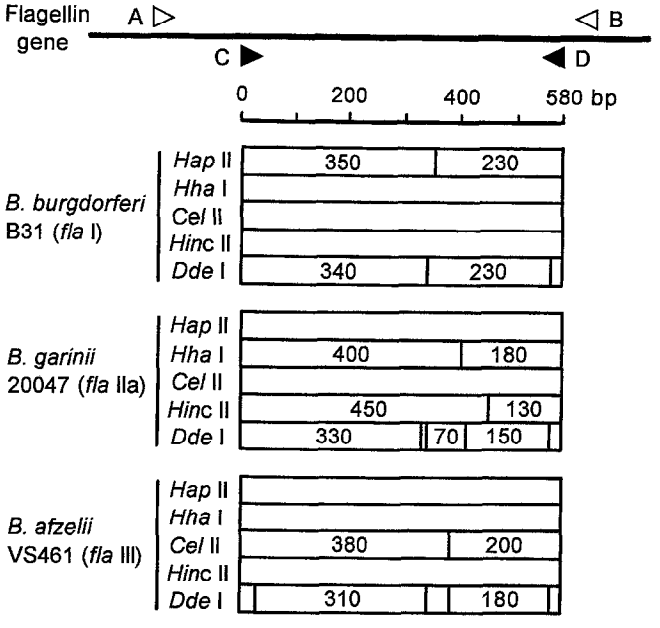

Figure 1. Schematic diagram of locations of PCR primer sets on the flagellin gene and restriction maps of amplified fragment. Open arrowheads indicate the external primers A and B for the first PCR. Closed arrowheads indicate the internal primers C and D for the second PCR. The flagellin (fla) types are given in parentheses. Lengths of cleaved fragments (in bp) discernible in 2.5% agarose gels are shown in the restriction maps. The maps are based on the sequences of the type strains (accession nos. of the GenBank/EMBL/DDBJ databases): B31 (X16933), 20047 (D82846), and VS461 (D63365).

The fla types I, II, and III correspond to *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively. Because the PCR-RFLP patterns of *B. garinii* were heterogeneous, the fla type II was divided into seven subtypes (designated subtypes IIa to IIg).

Clinical Samples

In cooperation with many dermatologists, human cutaneous biopsy samples were collected from tick-bite victims in Hokkaido and Nagano, Japan, where Lyme disease is endemic, during May to August in 1996. Based on the surveillance criterion of Centers for Disease Control and Prevention, a rash of 5 cm or larger was defined as erythema migrans.[27] Biopsy samples were taken from the peripheral reddish area of erythema. In some typical cases of erythema migrans, biopsy samples were taken from peripheral reddish and central non-reddish areas. Under sterile and cool conditions, the samples were transported to the laboratory within 1 to 2 days. The sample was divided in half and one piece was inoculated into BSK II medium; the remaining piece was preserved at -70°C until use for DNA preparation.

Confirmation of Species Identification

In human samples, the species status directly determined by nested PCR-RFLP analysis was verified by classifying the clinical isolates into ribotypes. The experimental conditions of ribotyping have been described.[4] The fla types also were determined in the clinical isolates, by previously published non-nested PCR-RFLP analysis.[16]

RESULTS

Sensitivity of Polymerase Chain Reaction

The sensitivity of the nested PCR system was evaluated (Table 1). Accurately prepared 10-mg earlobe pieces of normal mice and serial dilutions of cultured cells of *B. afzelii* N391 were used. For preparing template DNA,

Table 1. Sensitivity of Nested Polymerase Chain Reaction for Detection of Borrelial Flagellin Gene

Number of Borrelia *Cells Added to 10 mg Sample**	*Duplicate PCR*	
	First	*Second*
10^4	(+)/(+)	+/+
10^3	–/–	+/+
10^2	–/–	+/+
10	–/–	+/–
1	–/–	–/–
0	–/–	–/–

*The cells of *B. afzelii* N391 were added to normal murine earlobes for preparing template DNA.
+ = positive; (+) = weakly positive; – = negative.

Table 2. Detection of Borrelial Infections in Mice by Nested Polymerase Chain Reaction and Culture

		Positive (%)				
		PCR		Culture		
Borrelia Strains	*Number of Mice**	*Earlobe*	*Heart*	*Earlobe*	*Heart*	*Results of RFLP Typing†*
B. burgdorferi NCH-1	4	4 (100)	4 (100)	4 (100)	4 (100)	fla I
B. burgdorferi 297	5	5 (100)	5 (100)	5 (100)	5 (100)	fla I
B. garinii N346	4	4 (100)	4 (100)	3 (75)	3 (75)	fla IIa
B. garinii JEM6	7‡	7 (100)	7 (100)	5 (71)	7 (100)	fla IIe
B. afzelii N391	6	6 (100)	6 (100)	6 (100)	4 (67)	fla III
Uninfected	3	0 (0)	0 (0)	0 (0)	0 (0)	

*All mice were killed 3–5 weeks after infection for sampling earlobes and hearts. †All of the resultant PCR amplicons were examined by restriction fragment length polymorphism analysis. ‡Mice were infected by bites of nymphal ticks.
fla = flagellin.

each piece was lysed together with 1, 10, 10^2, 10^3, or 10^4 borrelial cells. Using those templates, the experiment of amplification was done in duplicate. In the first PCR, no positive signals were obtained in samples mixed with 1 to 10^3 cells; however, a weak positive signal of 800 bp was detected in a sample mixed with 10^4 cells. In the second

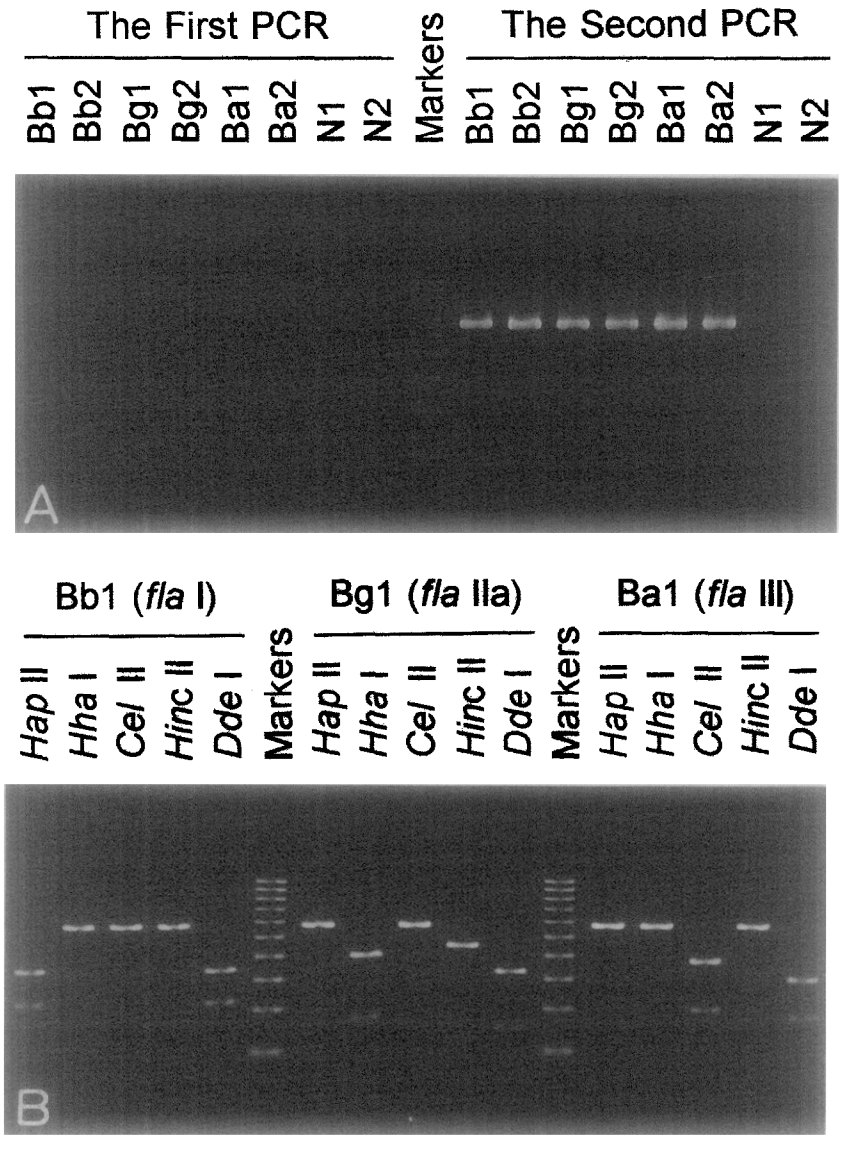

Figure 2. Nested PCR-RFLP analysis in mice infected with Lyme disease borreliae. *A*, Signal detection by the first and second PCR. Template DNA was prepared from earlobes. Markers, 100 bp ladder (100–1000 bp); Bb1 and Bb2, mice infected with *B. burgdorferi* 297; Bg1 and Bg2, mice infected with *B. garinii* N346; Ba1 and Ba2, mice infected with *B. afzelii* N391; N1 and N2, normal mice. The second PCR yielded products of 580 bp in length for all infected mice. *B*, RFLP of amplicons derived from samples of Bb1, Bg1, and Ba1. Lengths of DNA fragment (in bp) are as follows: Bb1 (*Hap*II, 350 and 230; *Hha*I, 580; *Cel*II, 580; *Hin*cII, 580; *Dde*I, 340 and 230), Bg1 (*Hap*II, 580; *Hha*I, 400 and 180; *Cel*II, 580; *Hin*cII, 450 and 130; *Dde*I, 330, 150, and 70), and Ba1 (*Hap*II, 580; *Hha*I, 580; *Cel*II, 380 and 200; *Hin*cII, 580; *Dde*I, 310 and 180). The flagellin (fla) types are indicated in parentheses.

PCR, a strong positive signal of 580 bp was constantly detected in samples mixed with 10^2 to 10^4 cells. In a sample mixed with 10 cells, one of the duplicated reactions was negative. Based on this result, the density of 10 to 10^2 cells per 10 mg cutaneous tissue was estimated to be the lower limit for detecting infections.

Amplification of Borrelial DNA in Infected Mice

Earlobe and heart tissues of mice experimentally infected with *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzeliti* were used to determine whether the PCR system was able to detect borrelial infections irrespective of differences in the causative species. The sensitivities for detecting infections between PCR and culture tests also were compared. All tissue samples used were taken from mice 3 to 5 weeks after infection. As summarized in Table 2, PCR showed positive results in all tissue samples from mice infected with each borrelial strain (*B. burgdorferi* sensu stricto NCH-1 and 297, *B. garinii* N346, and *B. afzelii* N391) by needle inoculation of cultured cells. Even in mice naturally infected with *B. garinii* JEM6 by tick bites, positive PCR signals were obtained in all tissue samples. Although the first PCR was ineffective in detecting signals, an approximately 580 bp fragment was amplified by the second PCR in all tissue samples of infected mice (Figure 2, *A*). The RFLPs of all amplicons obtained from the tissue samples were examined. The fla types directly determined in each tissue sample were completely consistent with those of *Borrelia* species infected in mice (see Table 2; Figure 2, *B*). The culture test was also excellent for detecting infections; however, its sensitivity was slightly lower than that of PCR.

Negative Conversion of Polymerase Chain Reaction Signals in Mice after Antibiotic Treatment

Mice naturally infected with *B. garinii* JEM6 by bites of infected nymphs, were used to determine whether the positive results of PCR were converted to negatives after antibiotic treatment. Twelve infected mice were used for this experiment. Of those, six mice were treated with intraperitoneal injection of ceftriaxone (Rocephin; Japan Roche Inc., Tokyo, Japan). The other six untreated mice served as positive controls. The antibiotic treatment started at 21 days after tick exposure. After taking biopsy samples from earlobes, the antibiotic (10 mg/d) was injected into each mouse for 5 consecutive days. The treated mice were killed at two different intervals (6 days and 41 days after treatment) for sampling earlobe and heart tissues. Necropsy samples were similarly collected from untreated mice. All biopsy and necropsy samples were subjected to both PCR and culture tests. As summarized in Table 3, negative conversion of PCR signals was observed in all treated mice. The negative result of culture tests in treated mice also indicated that borreliae were eliminated from murine tissues after antibiotic treatment.

Clinical Application of Polymerase Chain Reaction

The diagnostic value of the nested PCR system was evaluated by using human cutaneous biopsy samples taken from tick-bite victims in Japan. As shown in Table 4, 26 biopsy samples from 23 victims (designated patient 1 to 23) were subjected to both PCR and culture tests. Of those victims, 12 cases showed small erythematous lesions that were considered to be hypersensitivity reactions induced by tick bites. The other 11 cases (patient 2, 6, 8, 10, 13, 14, 15, 16, 20, 22, and 23) were clinically diagnosed as erythema migrans. Of those cases, the complication of facial palsy arose in patient 2. The remaining cases showed uncharacteristic symptoms, such as fatigue and fever. Patient 10, 13, and 22 showed typical erythema migrans characterized by an annular rash expanding centrifugally with central clearing. In those cases, we could examine two biopsy samples taken from peripheral reddish and central non-reddish areas.

Also in human samples, the first PCR was insensitive; however, an expected 580 bp fragment could be amplified by the second PCR (Figure 3, *A*). In the samples of small erythematous lesions, both PCR and culture tests were negative; however, positive results were obtained in several samples of erythema migrans (see Table 4). In three cases with typical erythema migrans, all biopsy samples from peripheral reddish and central non-reddish areas were positive for both tests. Of 11 cases of erythema migrans, borrelial infections were detected in seven

Table 3. Negative Conversion of Nested Polymerase Chain Reaction Signals in Mice Infected with *B. garinii* JEM6 after Antibiotic Therapy

	*Detection of Borrelial Infections**					
	Biopsy Samples		*Necropsy Samples*			
	PCR	*Culture*	*PCR*		*Culture*	
Groups of Mice†	*Earlobe*	*Earlobe*	*Earlobe*	*Heart*	*Earlobe*	*Heart*
Treated (6 days)	3/3	3/3	0/3	0/3	0/3	0/3
Untreated (6 days)			3/3	3/3	3/3	3/3
Treated (41 days)	3/3	3/3	0/3	0/3	0/3	0/3
Untreated (41 days)			3/3	3/3	3/3	3/3

*Number positive/number examined. †Antibiotic therapy started at 21 days after exposure to infected nymphal ticks. After taking biopsy samples from earlobes, mice were treated with ceftriaxone for 5 consecutive days. The treated and untreated mice were killed 6 days and 41 days after therapy.

Table 4. Clinical Application of Nested Polymerase Chain Reaction to Detect Borrelial DNA from Cutaneous Biopsy Samples of Tick-Bite Victims

			Test Results of Biopsy Samples		*Established Isolates*	
Patient	*Sex Age (y)*	*Clinical Diagnosis (Biopsy Site)*	*PCR (fla Type)*	*Culture*	*fla Type*	*Ribotype*
1	F (62)	Small erythema (P)	–	–		
2	F (63)	EM and facial palsy (P)	+ (IIa)	+	IIa	IIa
3	F (79)	Small erythema (P)	–	–		
4	M (47)	Small erythema (P)	–	–		
5	M (20)	Small erythema (P)	–	–		
6	F (4)	EM (P)	+ (IIa)	+	IIa	IVa
7	M (36)	Small erythema (P)	–	–		
8	F (75)	EM (P)	–	–		
9	F (70)	Small erythema (P)	–	–		
10	M (57)	EM (P)	+ (IId)	+	IId	IVa
		(C)	+ (IIa)	+	IIa	IVa
11	F (68)	Small erythema (P)	–	–		
12	M (56)	Small erythema (P)	–	–		
13	M (69)	EM (P)	+ (IId)	+	IId	IVa
		(C)	+ (IId)	+	IId	IVa
14	F (59)	EM (P)	+ (IIa)	+	IIa	IVa
15	F (63)	EM (P)	–	+	IIa	IIa
16	F (61)	EM (P)	+ (IIe)	+	IIe	IVa
17	F (61)	Small erythema (P)	–	–		
18	M (55)	Small erythema (P)	–	–		
19	F (60)	Small erythema (P)	–	–		
20	M (53)	EM (P)	–	–		
21	F (18)	Small erythema (P)	–	–		
22	F (47)	EM (P)	+ (IIa)	+	IIa	IVa
		(C)	+ (IIa)	+	IIa	IVa
23	F (55)	EM (P)	–	–		

F = female; M = male; EM = erythema migrans; P = peripheral part of erythema (reddish area); C = central part of erythema migrans (non-reddish area).

(64%) by PCR and in eight (73%) by culture. Fisher's exact probability test revealed that these values did not differ significantly (P = 0.5). All PCR-positive samples were also positive by culture. Each RFLP pattern of PCR amplicons was classified into the fla type IIa, IId, or IIe (see Table 4 and Figure 3, *B*). The results of fla typing of established isolates were completely consistent with the directly determined results in tissue samples. All of the fla types determined in human samples corresponded to *B. garinii*. Species identification by ribotyping classified each established isolate into the ribotype IIa or IVa according to the typing scheme described previously.[4] All ribotypes determined in this study also corresponded to *B. garinii*.

DISCUSSION

In northern Japan, the adult ticks of *I. persulcatus* aggressively attack humans and serve as a vector for Lyme disease. Several studies indicated that borreliae isolated from *I. persulcatus* were highly heterogeneous in their outer surface proteins, OspA and OspB.[12,28] Since the serotyping of Osp proteins seems to be insufficient for species identification, the ribotyping system for epidemiologic studies was developed.[4,5] The studies revealed that Japanese *I. persulcatus* harbors borreliae belonging to the ribotype II (*B. garinii*), III (*B. afzelii*), IV, V, or VI. Although the latter three ribotypes are regarded as unknown species,[4] a recent phylogenetic study on the flagellin gene demonstrated that those are intraspecies variants of *B. garinii*.[3] Most patients with Lyme disease in Japan show only symptoms of fever, malaise, and erythema migrans after tick bites, and severe multisystemic manifestations, such as cardiopathy, neuropathy, and arthropathy, are uncommon in comparison with European and North American Lyme disease.[23] A possible reason for the clinical difference relates to the specific *B. garinii* strains in Japan, although other factors, such as ethnic difference in patients cannot be ignored.

The authors previously reported that, in Japan, borreliae were easily isolated by using erythema migrans lesions of tick-bite victims as culture sources and most of the isolates were classified as *B. garinii*.[3,4,23] Confirmation of causative species seems to be unnecessary for diagnostic laboratories; however, differential diagnosis is still required because several *Borrelia* species whose pathogenicities are unknown are also distributed in Japan.[19,20] In vitro cultivation is the most reliable method for the direct demonstration of borreliae from human cutaneous tissues, although cultivation is time-consuming and the results vary among laboratories.[29,30] Species identification of borrelial isolates from clinical samples has been carried out mainly by Western blot (serotyping) or Southern blot (ribotyping) analysis.[1,2,4,12,14] However, both methods are also time-consuming. These

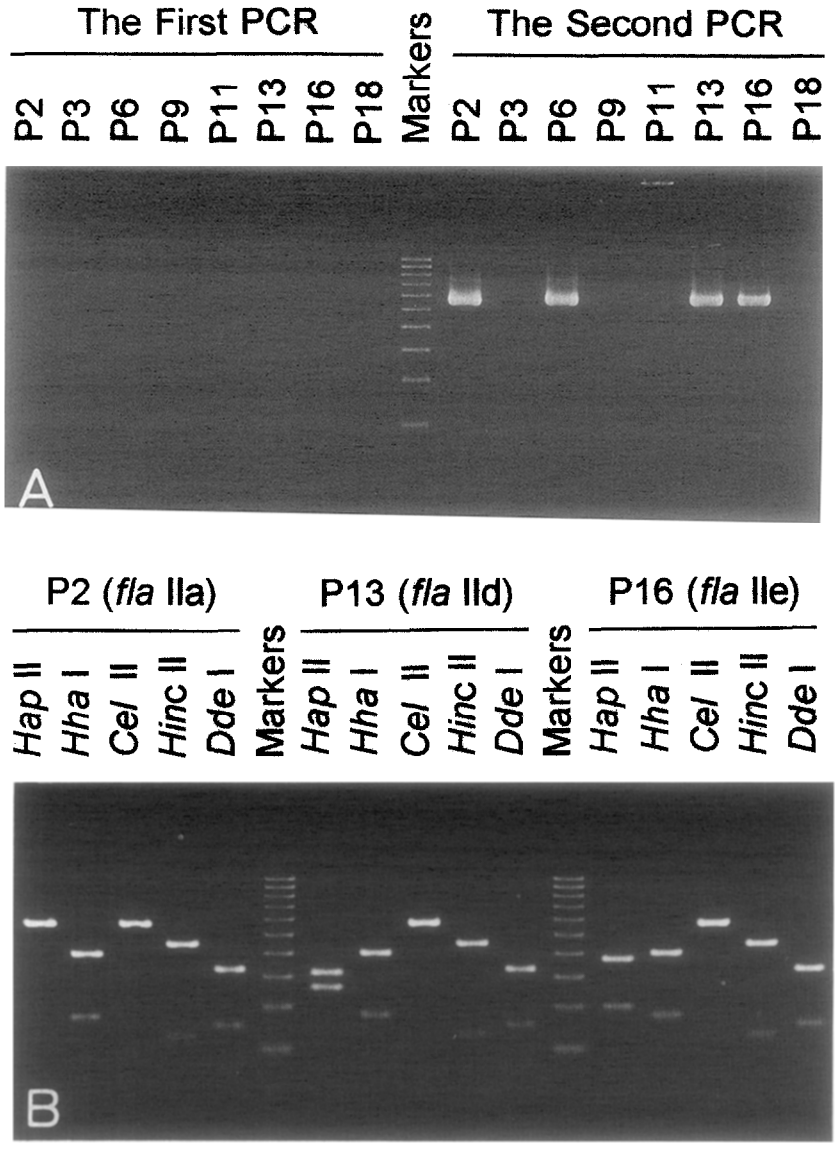

Figure 3. Clinical application of nested PCR-RFLP analysis for human cutaneous biopsy samples. *A*, Signal detection by the first and second PCR. Template DNA was prepared from erythematous lesions of tick-bite victims. Markers, 100 bp ladder (100–1000 bp); P2, P3, P6, P9, P11, P13, P16, and P18, samples from patients summarized in Table 4. P2, P6, P13, and P16 were clinically diagnosed as erythema migrans. The second PCR yielded products of 580 bp in length for samples of P2, P6, P13, and P16. *B*, RFLP of amplicons derived from samples of P2, P13, and P16. Lengths of DNA fragments (in bp) are as follows: P2 (*Hap*II, 580; *Hha*I, 400 and 180; *Cel*II, 580; *Hin*cII, 450 and 130; *Dde*I, 330, 150, and 70), P13 (*Hap*II, 320 and 260; *Hha*I, 400 and 180; *Cel*II, 580; *Hin*cII, 450 and 130; *Dde*I, 330, 150, and 70), and P16 (*Hap*II, 370 and 210; *Hha*I, 400 and 180; *Cel*II, 580; *Hin*cII, 450 and 130; *Dde*I, 330, 150, and 70). The flagellin (fla) types are indicated in parentheses.

situations prompted the authors to develop a simple and rapid PCR-RFLP assay for detecting borrelial DNA from skin biopsy samples and for directly identifying the causative species.

Various PCR primer sets have been devised to amplify DNA of Lyme disease borreliae.[31-39] Most of the primers were designed to be specific to the OspA or flagellin gene and have been used for clinical diagnosis. In the OspA gene-targeted PCR analysis,[31,40] not all strains of Lyme disease borreliae could be amplified, because the differences in the OspA gene sequence of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* are pronounced.[13,14] To overcome this defect, several species-specific primer sets have been designed.[7] However, the reactivities of those primer sets against other *Borrelia* species related to Lyme disease borreliae are unknown. On the other hand, the flagellin gene-targeted PCR can amplify borrelial DNA irrespective of differences in various geographic

strains,[40,41] because the flagellin gene is highly conserved among Lyme disease borreliae and their relatives.[13,16,38] Nevertheless, as shown in a previous report,[16] minor differences in the flagellin genes can be used to differentiate *Borrelia* species.

The density of borrelial cells in cutaneous and internal lesions is unknown; however, it seems to be low, because few borreliae were found in histopathologic specimens.[42] To amplify borrelial DNA from tissues and body fluids, several researchers used a single primer set in their early PCR systems, and the sensitivity and specificity were intensified by hybridization, using probes complementary to amplicons.[32,33,39,43,44] Recently, nested PCR has frequently been used as a highly sensitive test for the diagnosis of Lyme disease.[35,36,45-47] This method has the advantage that amplified borrelial DNA is easily discernible in ethidium bromide-stained agarose gels without intensifying the signals by hybridization. Therefore, in this study, this method was applied for amplifying the partial flagellin genes from tissue samples and its specificity was evaluated by analyzing the RFLP of the amplicons.

The nested PCR system was consistent, reproducible, and unaffected by vast excesses of host DNA. With regard to sensitivity, the authors estimated that the density of 10 to 10^2 borrelial cells per 10 mg cutaneous tissue was the lower limit for detecting infections. Therefore, a false-negative result may occur in this system if the number of borreliae present in the minute tissue sample is decreasing to a level of 1 to 10. The chromosome copy number per borrelial cell may also have influence on the sensitivity of this detection system. The number has not yet been reported for Lyme disease borreliae, but an investigation with the relapsing fever agent, *Borrelia hermsii*, revealed a relatively high value of 16 chromosome copies per cell for borreliae grown in mice.[48]

Earlobe and heart tissues of mice experimentally infected with *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii* were used to evaluate the sensitivity and specificity of the PCR system. Positive PCR results were obtained from all tissue samples irrespective of differences in the causative species. The PCR for murine tissues was slightly more sensitive than culture in detecting the presence of borreliae. Each RFLP pattern of PCR products completely corresponded to the causative species, indicating that the amplicons originated from borreliae present in the tissues. The diagnostic value of PCR has frequently been assessed by using animal models.[33,44-46] In most animal studies reported to date, borrelial infection was induced by needle inoculation of cultured cells; however, this infection does not accurately reflect the natural infection. Therefore, in this experiment, the authors included mice naturally infected with *B. garinii* by tick bites. Even in those mice, positive PCR signals were obtained.

The fate of borrelial DNA after antibiotic treatment was also examined in earlobe and heart tissues of mice infected with *B. garinii* by tick bites. As reported by Malawista et al,[49] the ability to amplify borrelial DNA quickly disappeared from tissues that had become culture-negative after antibiotic treatment. Although the in vivo kinetics for the clearance of dead borrelial cells is unknown, PCR amplification probably occurs if sufficient numbers of viable borrelial cells are present in tissue samples.

In clinical application of the nested PCR-RFLP system, the authors obtained satisfactory results concerning the sensitivity and specificity. Results of both PCR and culture tests were negative in all samples of erythematous lesions that were considered to be hypersensitivity reactions induced by tick bites. In lesions clinically diagnosed as erythema migrans, both PCR and culture tests frequently yielded positive results. All PCR-positive specimens also were positive by culture, and the causative species could be identified as *B. garinii* by analyzing the RFLP of tissue-derived amplicons. The apparent false-negative result by PCR occurred in a sample from patient 15, which showed a positive result by culture. This discrepancy may be due to the low density or inconsistent distribution of borreliae in the cutaneous samples. Histopathologic studies on Lyme disease demonstrated that distribution of borreliae was not uniform, but rather occurred in a multifocal pattern.[43,50] Interestingly, in patient 10, with typical erythema migrans, the fla type determined in the peripheral reddish lesion was different from that in the central non-reddish lesion. This case seems to have been infected with two different types of borreliae by the bite of a co-infected tick. Simultaneous infection of different *Borrelia* species in human Lyme disease has been reported in Europe.[7] Clearance of borrelial DNA in human cutaneous tissues following antibiotic treatment could be examined in patient 2. This case was treated with intravenous injection of ceftriaxone (2 g/d) for 10 days, and a biopsy sample was retaken from the initial biopsy site 18 days after therapy. Both PCR and culture tests for this sample yielded negative results, suggesting that the PCR analysis is applicable to evaluate the efficacy of antibiotic treatment.

CONCLUSION

The nested PCR-RFLP system could be used as an easy and reliable diagnostic tool for the detection and species identification of borreliae in human cutaneous biopsies. In this system, it took 2 days to complete the analysis. This rapidity is important for planning the antibiotic treatment in the early phase of infection. Because the devised primers are complementary to the conserved regions of the flagellin genes, this system may have universal application for clinical and epidemiologic uses in various localities of

the world. In the present study, the usefulness of this nested PCR-RFLP system for other *Borrelia* species (*B. japonica, B. tanukii, B. turdae*, and *B. andersonni* and the genomic groups PotiB2, VS116, and DN127) could not be determined; however, this system may apply to these species.

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