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Single-Tube Nested Polymerase Chain Reaction Assay Based on Flagellin Gene Sequences for Detection of *Borrelia burgdorferi* sensu lato

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An inherent drawback of nested PCR systems to increase sensitivity of PCR-based assays is that tubes must be opened after the first round of amplification in order to transfer template molecules to the second amplification reaction; this procedure introduces the risk of carry-over contamination of negative specimens. To obviate this disadvantage, a nested PCR assay for detection of *Borrelia burgdorferi* in which both amplifications are performed in a single tube that remains closed throughout the entire process was devised. The assay is based on flagellin gene sequences with previously determined species-wide and species-specific properties. The nested PCR system proved to be 1000 times more sensitive than the conventional assay. Using the nested PCR system, ten spirochaetes could be routinely detected by agarose gel electrophoresis alone, whereas the conventional PCR system could detect only 10^4 spirochaetes under these conditions. After Southern transfer of amplification products and hybridization with ^{32}P- or chemiluminescent-labeled probes, the nested PCR system could easily detect a single spirochaete by both means, whereas the sensitivity of the conventional PCR assay varied from 10^1 (^{32}P) to 10^3 (chemiluminescence) spirochaetes. This single-tube nested PCR system should be a useful addition to the current range of diagnostic assays for Lyme borreliosis.

Lyme borreliosis is a multisystem infection caused by spirochaetes of the genus *Borrelia* and acquired as a consequence of a bite from an infected tick of the *Ixodes ricinus* complex (1, 2). In Europe, at least three species are known to be causative agents of the disease: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (3). Although the earliest manifestations are usually cutaneous (erythema migrans is the pathognomonic lesion), the disease may progress to involve the joints, heart, nervous system or even eyes (4).

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Despite the distinctive appearance of typical erythematous eruptions, the clinical course is frequently atypical, and a diagnosis cannot always be made on clinical grounds alone. Currently, serologic tests are most often used as a laboratory aid, but sensitivity is often low and the results can be inconclusive (5). Culture of organisms can also be used for diagnosis, but does not always yield spirochaetes and often requires lengthy growth times varying from weeks to months. Moreover, Lyme borreliosis is a disease in which the response to treatment declines as the duration of illness increases. This makes a quick and accurate diagnosis and the institution of early antibiotic therapy highly advantageous. For these reasons, investigators have sought other methods of direct detection that are faster and more sensitive than culture.

The technique of in vitro amplification of DNA sequences using the polymerase chain reaction (PCR) was quickly exploited as a new means of directly identifying the presence of the spirochaete. PCR assays based on a number of well characterized borrelial genes have been de-

Table 1: *Borrelia* species and strains used to test PCR assay.

Species	Strain	Biological origin	Geographic location	Source
B. burgdorferi sensu stricto	B31 (ATCC 35210)	tick (scapularis)	USA	ATCC
B. garinii	PBi	cerebrospinal fluid	Germany	R.C. Johnson
B. afzelii	PGau	skin (ACA)	Germany	S.W. Barthold

ACA, acrodermatitis chronica atrophicans.

scribed (reviewed in 6). Several studies have used *Borrelia burgdorferi* sensu lato flagellin gene sequences as the basis for PCR amplification assays (7–13) and some investigators have devised nested PCR systems in an attempt to improve the sensitivity of the assay (14–17).

An inherent drawback of nested PCR amplification assays is the need to open tubes after the first round of amplification to transfer products to a second PCR amplification reaction that utilizes a different primer pair or to introduce new reagents and/or primers. This process carries with it the possibility of cross-contamination of negative specimens with amplicons derived from positive specimens during the first round of amplification. To circumvent this problem, we set out to devise a nested PCR amplification assay that can be performed in a single tube, which does not have to be opened until the amplicon analysis stage when the amplification reaction itself is complete. We have previously devised a PCR assay based on flagellin-gene sequences that was shown to detect a wide cross-section of *Borrelia burgdorferi* sensu lato isolates (18). We have used this system as the basis for our single-tube nested PCR assay. We describe here the design and development of the system, and present comparative data on the relative levels of sensitivity of the nested and conventional PCR assays.

Materials and Methods

Bacterial Strains and Growth Conditions. Reference strains of *Borrelia burgdorferi* sensu lato used in the development of the nested PCR assay are listed in Table 1. The sources of the strains have been described previously (18). All strains were grown in BSK II medium (19) modified by the addition of L-cysteine (1 mM) and dithiothreitol (1 mM) (20). Cultures were incubated at 33°C.

Clinical Specimens. Skin biopsy specimens from erythema migrans (EM) lesions were obtained from patients who presented to the University Medical Centre, Ljubljana, Slovenia. The tissue was placed in 500 µl of digestion buffer (25 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 1.2% Triton X-100) contained in 2 ml O-ring-sealed, screw-cap tubes (Sarstedt, USA) and then shipped to Chicago, USA, by express mail at ambient temperature for processing by PCR.

Skin biopsy specimens were prepared for PCR using a modification of the method described by Boom et al. (21). On receipt of the specimens in tissue digestion buffer, proteinase K was added to a final concentration of 1.5 mg/ml and the tubes incubated at 55°C overnight. After dissolution of the skin tissue, 1 ml of L2 buffer (21) was added and the tubes incubated at 65°C for 15 min. Eighty µl of Celite suspension (21) was then added and the tubes placed on a rotating mixer for 1 h at room temperature. The Celite was then pelleted by centrifugation and washed sequentially with L2 buffer (2 times), 70% ethanol (2 times) and 100% ethanol. The Celite pellet was then dried in a 65°C heating block for 15 min and resuspended in 100 µl of Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). DNA was allowed to elute from the Celite at 55°C for 1 h, the Celite was pelleted by centrifugation and the supernatant transferred to a new tube. Patient specimens prepared for PCR analysis were stored at −70°C until needed. In control experiments, no inhibition of PCR amplification was observed when specimens were prepared using this protocol. However, after re-amplification of specimens stored at −70°C, the signal obtained often appeared weaker than that obtained after the first amplification.

Polymerase Chain Reaction Primers and Probes. The oligonucleotide primers and probes used in this study are listed in Table 2. For experiments involving the development of the assay and utilizing three species of *Borrelia burgdorferi* sensu lato, the oligonucleotide sequences appropriate to each species were used. For the investigation of clinical specimens, where the infecting species was unknown, all three sets of primers were routinely included in amplification reactions to further increase the sensitivity of detection. The primer concentrations used were: FL3b, FL3g, FL3a, FL5b, FL5g, FL5a, 0.0033 µM each; FL6b, FL6g, FL6a, FL7b, FL7g, FL7a, 0.33 µM each. Oligonucleotide probes for the three species (FL8, FL15 and FL16) were also mixed in equal proportions after labeling with ^{32}P.

For the preparation of chemiluminescent (ECL)-labeled probe, PCR primers FL1 and FL2 were used to amplify an internal 220 bp fragment of the flagellin gene of *Borrelia burgdorferi* sensu stricto strain B31. This fragment was then purified from a 2% low-melting temperature agarose gel by phenol extraction and ethanol precipitation (24). The purified fragment was labeled using an ECL kit (Amersham, USA) in accordance with the manufacturer's recommended procedures. Oligonucleotide probes (FL8, FL15, FL16) were 5' end-labeled using $\gamma^{32}P$ ATP and T_4 polynucleotide kinase as described previously (18).

Design of the Single-Tube Nested Polymerase Chain Reaction Amplification System. The design is shown diagrammatically in Figure 1. The system makes use of different regions of the flagellin gene sequence with different G + C contents from which primers with vastly different optimum annealing temperatures were chosen. The system comprises an outer set of primers (FL3 and FL5) that are 31 and 32 base pairs (bp) long

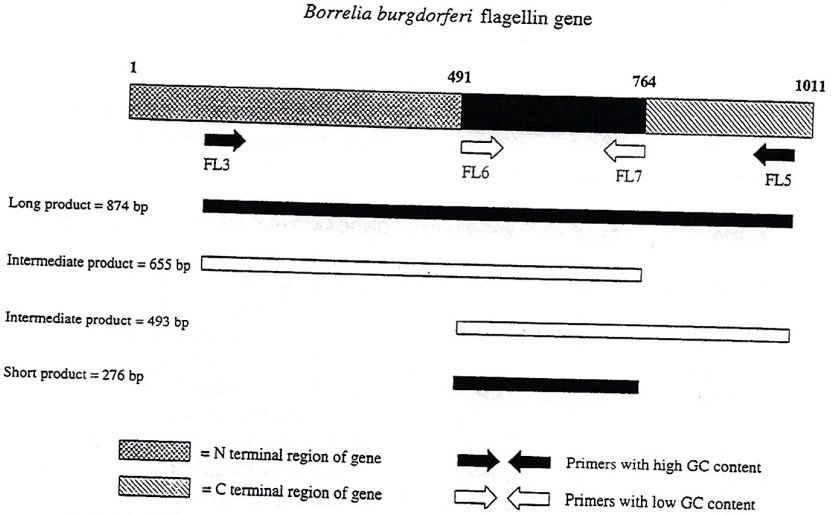

Figure 1: Diagrammatic representation of the single-tube nested PCR system. The *Borrelia burgdorferi* flagellin gene is shown (1011 bp) with the location of the primer pairs and all possible amplification products. Nucleotide numbers are based on the sequence of Gaßman et al. (22).

and have a high G + C content. These primers anneal and prime the amplification of an 874 bp fragment (the long product) at an annealing temperature of 70°C. The internal set of primers (FL6 and FL7) have been described previously (18). They are substantially shorter (25 bp) and have a much lower G + C content. They prime the amplification of a 276 bp fragment (the short product) at an annealing temperature of 54°C but, at the 70°C annealing temperature, these primers are at a disadvantage in the annealing reaction.

Both sets of primers are added together at the start of the amplification reaction along with buffers, nucleotides, polymerase and template. The tube is then sealed and the amplification started. During the first 30 cycles of amplification the annealing temperature is kept high (70°C), only the outer set of primers anneal and the long product is synthesized. After 30 cycles, the outer primers are substantially depleted. During the next 30 cycles, the annealing temperature is lowered to 54°C, the internal primers FL6 and FL7 anneal to the long product, and the short product is synthesized. It can be seen from Figure 1 that the possibility of synthesizing two products of intermediate length also exists (produced by primers FL3/FL7 and FL6/FL5). The system was therefore optimized to reduce amplification of the intermediate products.

Conventional Polymerase Chain Reaction Amplification. This was performed as described previously (18) except that the final concentration of gelatin in the amplification buffer was increased from 0.001 to 0.005%. Primer pairs FL6 and FL7 were used at a final concentration of 1 μM. Amplification reactions routinely contained 5 U of Uracil-N-glycosylase (Amperase, Perkin-Elmer-Cetus, USA) and 400 μM uracil triphosphate instead of deoxythymidine triphosphate. For this reason, the previously described denaturation, annealing and amplification conditions were preceded by incubation of PCR assays at 22°C for 10 min followed by denaturation at 94°C for 12 min. For purposes of comparison with nested PCR, 60 cycles of conventional PCR amplification were used. Conventional PCR, without addition of new reaction components, gives a maximal yield of product after 45 cycles; however, no net loss of product was seen after a further 15 cycles.

Nested Polymerase Chain Reaction Amplification. This was performed using the same amplification buffer as in conventional PCR except that a Mg^{2+} concentration of 2.0 mM was found to be optimal for nested PCR (see Results). Amplification reactions also contained primer pairs FL6 and FL7 at a final concentration of 1 μM and primer pairs FL3 and FL5 at a final concentration of 0.01 μM (see results section for optimization of primer pair ratios). For nested PCR amplifications, the following incubation times, temperatures, and cycle parameters were used: 22°C for 10 min (uracil-N-glycosylase excision of uracil bases); 94°C for 12 min (uracil-N-glycosylase inactivation and denaturation) followed by 30 cycles of amplification at 94°C for 1 min (denaturation), 70°C for 2 min (high-temperature annealing), and 72°C for 3 min (extension); followed by a further 30 cycles of amplification at 94°C for 1 min (denaturation), 54°C for 2 min (low-temperature annealing), and 72°C for 3 min (extension); followed by a final extension at 72°C for 7 min.

Analysis of Amplification Products. For the analysis of PCR amplification products, aliquots of amplification reactions were applied to 2% agarose gels and electrophoresed for 4 h at 90 V. Preparation of total genomic DNA from *Borrelia burgdorferi* sensu lato strains, ethidium bromide staining of gels, photography, Southern transfer procedures, hybridization with radioactively labeled oligonucleotide probes, washing and autoradiography were all performed as described previously (18). Hybridization with ECL-labeled chemiluminescent probes, washing and exposure of films were performed as recommended by the manufacturer (Amersham).

Optimization of Primer-Pair Ratios. For the optimization of the above parameters, nested PCR amplification assays were

Table 2: Nucleotide sequences of PCR primers and probes.

Species	Oligo-nucleotide	Nucleotide numbers[a]	Sequence
B. burgdorferi	FL1[b]	520–540	5' AGAGTTCATGTTGGAGCAACC 3'
B. burgdorferi	FL2[b]	717–737	5' GCATCAACTGTAGTTGTAACA 3'
B. burgdorferi	FL3b	112–142	5' CGAGCTTCTGATGATGCTGCTGGCATGGGAG 3'
B. garinii	FL3g[c]	112–142	5' AGAGCTTCTGATGATGCTGCTGGTATGGGGG 3'
B. afzelii	FL3a[c]	112–142	5' CGAGCTTCTGATGATGCTGCTGGTATGGGGG 3'
B. burgdorferi	FL5b	954–985	5' GGGGAACTTGATTAGCCTGCGCAATCATTGCC 3'
B. garinii	FL5g[c]	954–985	5' GGGGAACTTGATTAGCTTGCGCAATCATTGCC 3'
B. afzelii	FL5a[c]	954–985	5' GAGGAACTTGATTAGCCTGTGCAATCATTGCC 3'
B. burgdorferi	FL6b	492–516	5' TTCAGGGTCTCAAGCGTCTTGGACT 3'
B. garinii	FL6g[c]	492–516	5' TTCAGGATCTCAAGCTTCTTGGACC 3'
B. afzelii	FL6a[c]	492–516	5' TTCAGGATCTCAAGCTTCTTGGACT 3'
B. burgdorferi	FL7b	743–767	5' GCATTTTCAATTTTAGCAAGTGATG 3'
B. garinii	FL7g[c]	743–767	5' GCATTTTCTATTTTAGCAAGAGATG 3'
B. afzelii	FL7a[c]	743–767	5' GCATTTTCTATTTTAGCAAGTGATG 3'
B. burgdorferi	FL8[d]	594–642	5' CTCTGGTGAGGGAGCTCAAACTGCTCAGGCTGCACCGGTTCAAGAGGGT 3'
B. garinii	FL15[d]	594–641	5' CTCTGGTGAAGGAGCTCAGGCTGCTCAGACTGCACCTGTTCAAGAAGG 3'
B. afzelii	FL16m[d,e]	594–644	5' TGCTGGTGAGGGAGCTCAAGCTGCTCAGGCTGCACCTGTTCAAGAGGGTGC 3'

[a]Nucleotide numbers are based on the sequence of Gaßman et al. (22); [b]primers used for manufacture of a chemiluminescent probe; [c]sequence derived by Jauris-Heipke et al. (23); [d]^{32}P-labeled oligonucleotide probes; [e]modified from Picken (18).

set up using 1 ng of purified *Borrelia burgdorferi* sensu lato DNA as template and various primer pair ratios. At a ratio of 1:1, reactions contained 100 pmol each of FL3/FL5 and 100 pmol each of FL6/FL7. For a ratio of 1:10, reactions contained 10 pmol each of FL3/FL5 and 100 pmol each of FL6/FL7. For a ratio of 1:100, reactions contained 1 pmol each of FL3/FL5 and 100 pmol each of FL6/FL7. The relative amounts of long, short and intermediate amplification products were gauged by agarose gel electrophoresis.

Optimization of Magnesium Ion Concentration. Nested PCR amplification assays were set up using various numbers of intact spirochaetes of strain B31 (7.6×10^4, 10^4, 10^3, and 10^2) as template material and PCR buffers containing 1.5, 2.0, and 4.0 mM Mg^{2+} concentrations. The degree of amplification obtained and the size of amplification products were monitored by agarose gel electrophoresis.

Determination of the Relative Sensitivity of the Conventional and Nested Polymerase Chain Reaction Assays. Borrelia burgdorferi sensu stricto strain B31 was grown in BSK II medium until the medium turned yellow. An aliquot was taken from the top of the culture, diluted 20-fold in sterile phosphate-buffered saline (pH 7.0) and vortexed extensively to break up clumps of spirochaetes. The spirochaetal cell density was determined by dark-field microscopy using a Petroff-Hausser counting chamber. The culture was then diluted to 10^6/ml in sterile Tris-EDTA buffer, and a ten-fold dilution series prepared in the same diluent, containing 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 spirochaetes/ml. Ten µl aliquots of the dilutions were then added to conventional and nested PCR amplification assays such that they contained 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1}, and 10^{-2} spirochaetes/100 µl reaction. For PCR amplification assays nominally containing one spirochaete (10^0), both nested and conventional PCR assays were set up in quintuplicate to allow for sampling bias in the addition of the 10 µl aliquot. To allow for errors in determining the initial spirochaete concentration by counting, the PCR amplifications nominally containing 10^1 and 10^{-1} spirochaetes were also set up in quintuplicate. Negative controls contained 10 µl of Tris-EDTA buffer. Primer sequences were based on the B31 flagellin gene sequence (Table 2). Amplification reactions were placed in separate, identical thermocyclers and subjected to 60 cycles of amplification according to either the nested or conventional protocol. Twenty µl aliquots of the amplification products from both conventional and nested PCR assays were then analyzed by agarose gel electrophoresis and Southern hybridization as described above.

Comparison of the Conventional and Nested Polymerase Chain Reaction Assays Using Patient Specimens. To directly compare the two PCR amplification assays using clinical specimens, six samples prepared as described above and displaying a range of signal intensities after amplification were selected from a pool of known positive samples identified using the conventional PCR assay and a ^{32}P-labeled oligonucleotide probe. Twenty µl aliquots of these samples were added to conventional and nested PCR assays and subjected to 60 cycles of amplification according to either the nested or conventional protocol. After amplification, 20 µl aliquots were analyzed by agarose gel electrophoresis and Southern hybridization using an ECL-labeled chemiluminescent probe.

Results

Optimization of Primer-Pair Ratios. Nested PCR amplification assays were set up as described in the methods section using template DNA from each of the three species associated with Lyme borreliosis [*Borrelia burgdorferi* sensu stricto (strain B31), *Borrelia garinii* (strain PBi), and *Borrelia afzelii* (strain PGau)]. For each strain, primer se-

quences derived from the appropriate species were used (Table 2). Figure 2A shows the results of amplification using three primer-pair ratios: 1:1, 1:10 and 1:100. It can be seen that a primer pair ratio of 1:100 was optimal for the suppression of intermediate product amplification and produced the best yield of the desired 276 bp short product. This primer-pair ratio was therefore used in all subsequent experiments.

It is apparent from Figures 2 and 3 that synthesis of the 493 bp intermediate product (from primers FL6 and FL5) greatly exceeded that of the 655 bp intermediate product (from primers FL3 and FL7). This was presumably due to the fact that FL7 has a much lower G + C content (32%) than primer FL6 (52%), making it unstable at the 70°C annealing temperature.

Optimization of Magnesium Ion Concentration. Experiments designed to determine the optimum magnesium concentration of the PCR buffer for nested PCR were set up as described in the methods section, using the optimum primer pair ratio of 1:100. The primer sequences used were based on the B31 flagellin gene sequence (Table 2). Figure 2B shows the results of amplification reactions set up with 1.5, 2.0, and 4.0 mM magnesium and 7.6×10^4, 10^4, 10^3, or 10^2 strain B31 spirochaetes. Although a magnesium concentration of 1.5 mM was previously found to be optimal for the conventional flagellin gene PCR (18), it is apparent from Figure 2B that 2.0 mM is optimal for the nested PCR system.

Specificity of the Nested Polymerase Chain Reaction System. In addition to the representative isolates of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii* listed in Table 1, the specificity of the system was tested using a previously described panel of *Borrelia* species and strains (18). The results were the same as described for the conventional PCR assay (18) (data not shown). Also, strains of *Borrelia burgdorferi* sensu lato derived from Slovenian ticks (25) were tested, including *Borrelia garinii* isolates of MLg classes 1 to 6 (26) (data not shown). All of these strains gave rise to an amplification product of 276 bp.

Determination of the Sensitivity of the Nested Polymerase Chain Reaction System. This was determined using a model system, in which serial tenfold dilutions of spirochaetes were used in both nested and conventional PCR amplification assays, as described in the methods section. After amplification, products were separated by agarose gel electrophoresis and visualized by staining with

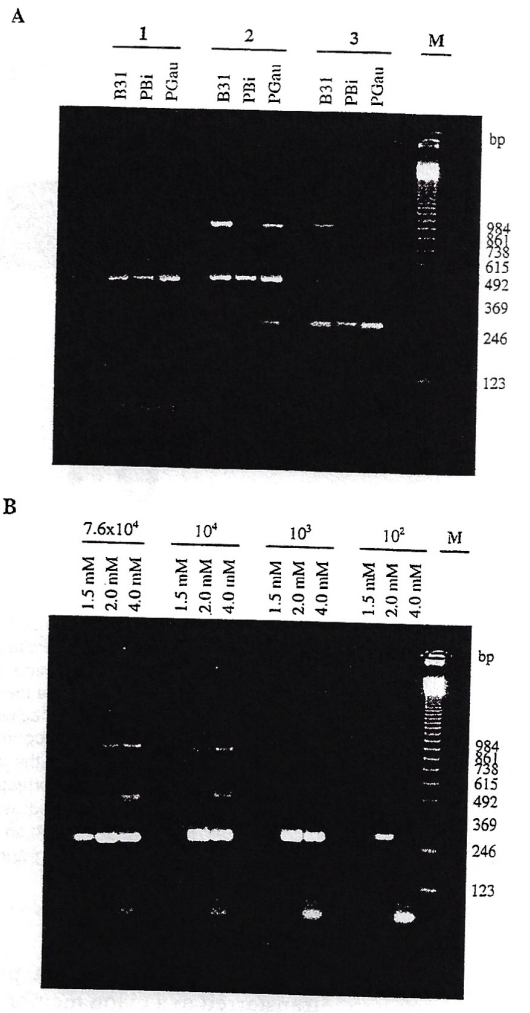

Figure 2: (**A**) Results of agarose gel electrophoresis of nested PCR amplification reactions set up with template DNA from each of the strains listed and containing different ratios of the primer pairs (FL3/FL5:FL6/FL7). 1, FL3/FL5 = 100 pmol, FL6/FL7 = 100 pmol (ratio 1:1); 2, FL3/FL5 = 10 pmol, FL6/FL7 = 100 pmol (ratio 1:10); 3, FL3/FL5 = 1 pmol, FL6/FL7 = 100 pmol (ratio 1:100); M, 123 bp ladder molecular size marker. (**B**) Optimization of magnesium concentration. Nested PCR assays were set up using a primer pair ratio of 1:100, containing 7.6×10^4, 10^4, 10^3, or 10^2 spirochaetes and different concentrations of magnesium as shown.

ethidium bromide. After agarose gel electrophoresis, a band corresponding in size to the expected 276 bp fragment could be seen on both conventional and nested PCR. Conventional PCR amplification detected 10^4 spirochaetes by this means; using the nested PCR amplification system, 10 spirochaetes could be detected routinely (data not

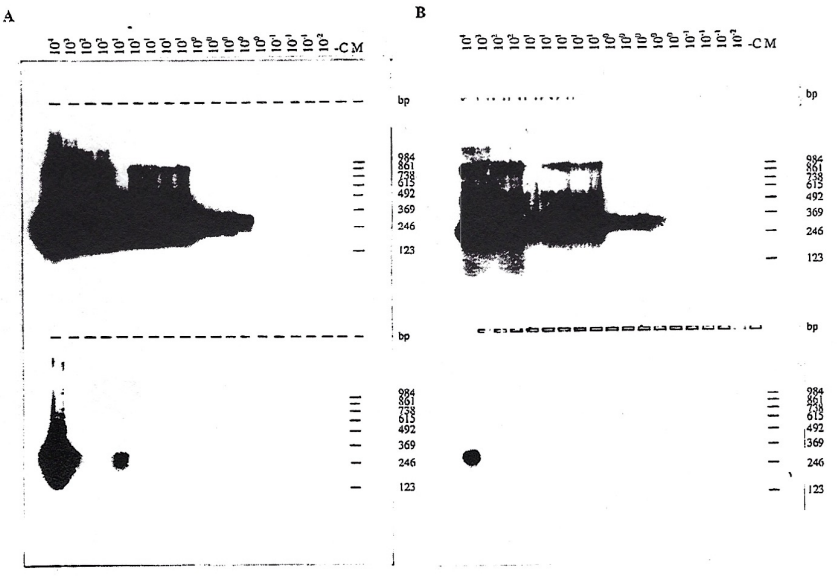

Figure 3: Comparison of the sensitivity of the nested and conventional PCR flagellin gene assays. (**A**) Results of agarose gel electrophoresis of amplification products, Southern transfer and ^{32}P-labeled oligonucleotide probe detection. The top half of the gel shows the results of nested PCR amplification, and the lower half the results of conventional PCR. Amplification reactions, both nested and conventional, were set up containing different dilutions of spirochaetes as shown above each lane. C, negative control containing only Tris-EDTA buffer as template. M, 123 bp ladder molecular size marker. In the lower half of the figure (conventional PCR) a band was visible on the original autoradiograph corresponding to the third replicate of 10^1 spirochaetes. (**B**) As in (**A**), but PCR products were detected using an ECL-labeled chemiluminescent probe. In the lower half of the figure (conventional PCR) an amplification product corresponding to 10^3 spirochaetes was visible on the original autoradiograph.

shown). The amplification products were also transferred to a nylon membrane by the method of Southern (27), and probed using both ^{32}P-labeled and chemiluminescent probes. The results of these experiments are shown in Figure 3. It is apparent from Figure 3 that, using a ^{32}P-labeled oligonucleotide probe, conventional PCR amplification could detect in the range of 10–100 spirochaetes whereas the nested PCR reaction could easily detect a single spirochaete (Figure 3A). In these experiments, it was sometimes difficult to obtain a smooth dilution series because of the well-known propensity of *Borrelia* spirochaetes to form clumps. The incongruously strong signal obtained at a dilution of 10^1 spirochaetes in the lower half of Figure 3A is presumably due to the detection of just such a clump. Figure 3B shows that, using the chemiluminescent probe, conventional PCR detected only 10^3 spirochaetes (a weak band was visible on the original autoradiograph), whereas nested PCR was still able to easily detect a single spirochaete with a strong signal.

Comparison of the Nested and Conventional Assays Using Patient Specimens. To test the assay using patient samples, six skin biopsy specimens that were known to be positive by conventional PCR and probing using a ^{32}P-labeled oligonucleotide probe were retested using both the conventional and nested PCR assays. Specimens were chosen that displayed a range of signal intensities from strong to the very weakest that we were able to detect. Aliquots of DNA preparations from these samples that had been stored at −70°C were re-amplified and, after agarose gel electrophoresis and Southern transfer, the blot was probed using a chemiluminescent probe. The results of this experiment are shown in Figure 4; while only three of the six specimens were positive by conventional PCR, all six specimens were positive by nested PCR.

Discussion

Nested PCR amplification systems have found an increasing application in the direct detection of *Borrelia burgdorferi* sensu lato (14, 15, 28–36). Most recently, several studies have described their use in the investigation of patient samples and experimentally infected animals (16, 17, 37, 38), and two of these latter studies (16, 17) utilized the FL6 and FL7 flagellin gene primer sequences that we have described previously (18). The advantages of nested PCR systems include (i) vastly increased sensitivity, and (ii) dilution of inhibitory compounds that could be present in the clinical sample. Unfortunately, the technique also has a major disadvantage associated with the possibility of carry-over contamination of negative specimens with amplicons derived from positive amplification reactions. Also, because of the two-step nature of nested PCR, decontamination procedures designed to prevent this, such as the incorporation of uracil-N-glycosylase or psoralen in PCR reactions, cannot be used.

We have devised a nested PCR system that eliminates the opening of tubes between the two stages of amplification and also permits the use of carry-over contamination-prevention procedures. Thus, the amplification reactions used to generate the data of Figure 4 included the use of uracil-N-glycosylase as a contamination preventative. We have also described the optimization of the assay and its use in the investigation of patient skin specimens. The current nested PCR assay is derived from our original conventional PCR assay, which was developed using primer and probe sequences from the conserved flagellin gene of *Borrelia burgdorferi* sensu lato (8, 22). These sequences have been demonstrated to detect a wide cross-section of European and North American *Borrelia burgdorferi* sensu lato isolates without cross-reacting with other closely related relapsing fever spirochaetes (18). The outer primer set (FL3/FL5) utilizes sequences from highly-conserved regions of the flagellin gene (18) and is therefore genus-specific. However, since the concentration of these outer primers is only 0.01 μM, the 874 bp fragment synthesized by them does not contribute significantly to the overall yield of amplification products; the 874 bp product serves only to boost the second stage amplification reaction. The inner primer set is specific for *Borrelia burgdorferi* sensu lato (18). The overall nested PCR assay thus retains the same 'species-wide' and 'species-specific' properties as that described previously (18). The use of PCR assays based on flagellin gene sequences may continue to be an important consideration in the detection of European isolates of *Borrelia burgdorferi* sensu lato, which are highly variable in the amino acid sequence of their outer-surface proteins.

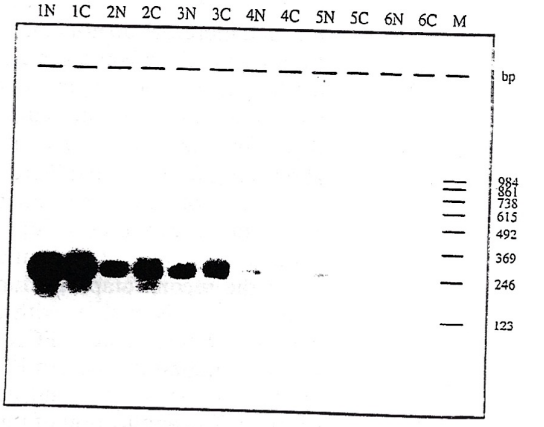

Figure 4: Comparison of nested and conventional PCR in the investigation of clinical specimens. This experiment shows the chemiluminescent detection of a range of skin biopsy specimens (from strongly positive to very weakly positive) previously identified by ^{32}P detection. The results for six different skin biopsy specimens are shown: lanes suffixed N show the results of nested PCR amplification, lanes suffixed C show conventional PCR results. M, 123 bp ladder molecular size marker. On the original autoradiograph a band was visible in lane 6N.

Since complete flagellin gene sequences from all three European species of *Borrelia burgdorferi* sensu lato (3) are now available (23), we used primers based on these sequences to further enhance the sensitivity of detection. However, it should be noted that the sequences are sufficiently similar for amplification to proceed using *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* or *Borrelia afzelii* DNA as template and primer sequences based on only one species (18).

The one possible advantage that conventional, double-tube nested PCR assays may possess over the system described here, is in the matter of dilution of *Taq* I polymerase inhibitors during transfer of amplification products from the first stage of amplification to new reactions in the second stage of amplification. However, in cases where inhibitors are not removed by an adequate sample preparation procedure, it may be envisaged that they will have a detrimental effect on the first amplification reaction and reduce the overall sensitivity of detection. We have found the sample preparation procedure of Boom et al. (21) to

be completely effective in removing PCR inhibitors and therefore do not regard this as a problem. In our view, the advantages associated with eliminating the possibility of carry-over contamination more than outweigh this single, minor disadvantage. For example, in one of the studies cited above (16), where nested PCR was used for the investigation of patient specimens, contamination-prevention procedures included the use of a dedicated biological containment cabinet for the set-up of the second amplification reaction and the cleaning of this cabinet with an aqueous hypochlorite solution between PCR runs. We anticipate that our single-tube, nested PCR assay will obviate these somewhat tedious, albeit necessary, precautions. The application of this assay to our own work, on the PCR diagnosis of Lyme borreliosis has freed us from the routine use of ^{32}P-labeled probes, without there being any concomitant loss in the sensitivity of detection.

A number of publications have reported on the sensitivity of both conventional (8–13) and double-tube nested PCR assays (15–17) based on flagellin gene sequences. Some of these studies used serial dilutions of purified DNA (8, 10, 12, 13, 17), some used dilutions of intact spirochaetes (9, 11, 15), and others used both (10, 13, 17). The sensitivity threshold was reported for two detection methods: (i) agarose gel electrophoresis and ethidium bromide staining of amplification products, and (ii) probing with ^{32}P-labeled probes. Results showed wide variations.

Using conventional PCR, purified DNA and agarose gel electrophoresis, the sensitivity threshold varied from 200 fg DNA (equivalent to 200 spirochaetes) (8) to 10 fg DNA (equivalent to 10 spirochaetes) (10, 12), and using ^{32}P detection, from 12.5 fg DNA (12–13 spirochaetes) (8) to 2 fg DNA (2 spirochaetes) (10). Using the same methods but with intact spirochaetes, the sensitivity threshold varied from 10 spirochaetes or fewer (11) to 2–3 spirochaetes (10) on agarose gel electrophoresis, and from 50 spirochaetes (9) to 10 spirochaetes (13) on ^{32}P detection. Our findings with regard to the sensitivity threshold of conventional flagellin-gene PCR using intact spirochaetes were lower. Thus, the lower limit of detection using agarose gel electrophoresis was 10^4 spirochaetes. Using Southern transfer and a ^{32}P-labeled oligonucleotide probe we could detect 10–100 spirochaetes. In these and other experiments, we have typically found that ^{32}P detection is approximately 100 to 1000 times more sensitive than agarose gel electrophoresis and ethidium bromide staining alone. Since in our hands chemiluminescent-labeled probes have not been as sensitive as ^{32}P-labeled probes, the limit of detection using this method in conventional PCR was only 10^3 spirochaetes. For this reason some of the skin specimens amplified using conventional PCR and shown in Figure 4 did not generate a detectable signal on chemiluminescence.

Using double-tube nested PCR systems, the reported sensitivity threshold for agarose gel electrophoresis has varied from < 10 spirochaetes (15) to < 5 spirochaetes (16) and for ^{32}P detection a 10 spirochaete level of sensitivity was reported for both intact spirochaetes and purified DNA (17). Sensitivity results for our single-tube nested PCR system compare favorably with these reported results. On agarose gel-electrophoresis alone we could typically detect 10 spirochaetes, and using Southern transfer and either a ^{32}P-labeled or chemiluminescent-labeled probe we could easily detect a single spirochaete.

In conclusion, the single-tube nested PCR flagellin gene assay described provides improved sensitivity over conventional flagellin gene PCR without the accompanying risk of carry-over amplicon contamination. The increase in the sensitivity of detection of nested PCR over conventional PCR using three different methods of detection was (i) for agarose gel electrophoresis from 10^4 spirochaetes to 10^1 spirochaetes; (ii) for chemiluminescent detection from 10^3 spirochaetes to 1 spirochaete; (iii) for ^{32}P detection from 10^1–10^2 spirochaetes to 1 spirochaete. The use of conserved flagellin gene sequences for primers and probes averts the possibility of failing to detect positive specimens as a result of sequence variation in polymorphic outer surface protein genes.

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References

1. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP: Lyme disease – a tick-borne spirochetosis? Science 1982, 216: 1317–1319.

2. Johnson RC, Schmid GP, Hyde FW, Steigerwalt AG, Brenner DJ: *Borrelia burgdorferi* sp. nov.: etiological agent of Lyme disease. International Journal of Systematic Bacteriology 1984, 34: 496–497.
3. Baranton G, Postic D, Saint Girons I, Boerlin P, Piffaretti JC, Assous M, Grimont PAD: Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. International Journal of Systematic Bacteriology 1992, 42: 378–383.
4. Steere AC: Lyme disease. New England Journal of Medicine 1989, 321: 586–596.
5. Berglund J, Eitrem R, Ornstein K, Lindberg A, Ringner Å, Elmrud H, Carlsson M, Runehagen A, Svanborg C, Norby R: An epidemiologic study of Lyme disease in southern Sweden. New England Journal of Medicine 1995, 333: 1319–1324.
6. Nocton JJ, Dressler F, Rutledge BJ, Rys PN, Persing DH, Steere AC: Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. New England Journal of Medicine 1994, 330: 229–234.
7. Persing DH, Telford SR, Rys PN, Dodge DE, White TJ, Malawista SE, Spielman A: Detection of *Borrelia burgdorferi* DNA in museum specimens of *Ixodes dammini* ticks. Science 1990, 249: 1420–1423.
8. Wallich R, Moter SE, Simon MM, Ebnet K, Heiberger A, Kramer M: The *Borrelia burgdorferi* flagellum-associated 41-kilodalton antigen (flagellin): molecular cloning, expression and amplification of the gene. Infection and Immunity 1990, 58: 1711–1719.
9. Jaulhac B, Nicolini P, Piemont Y, Monteil H: Detection of *Borrelia burgdorferi* in cerebrospinal fluid of patients with Lyme borreliosis. New England Journal of Medicine 1991, 324: 1440.
10. Lebech A-M, Hindersson P, Vuust J, Hansen K: Comparison of in vitro culture and polymerase chain reaction for detection of *Borrelia burgdorferi* in tissue from experimentally infected animals. Journal of Clinical Microbiology 1991, 29: 731–737.
11. Krüger WH, Pulz M: Detection of *Borrelia burgdorferi* in cerebrospinal fluid by the polymerase chain reaction. Journal of Medical Microbiology 1991, 195: 98–102.
12. Luft BJ, Steinman CR, Neimark HC, Muralidhar B, Rush T, Finkel MF, Kunkel M, Dattwyler RJ: Invasion of the central nervous system by *Borrelia burgdorferi* in acute disseminated infection. Journal of the American Medical Association 1992, 267: 1364–1367.
13. Lebech AM, Hansen K: Detection of *Borrelia burgdorferi* DNA in urine samples and cerebrospinal fluid samples from patients with early and late Lyme neuroborreliosis by polymerase chain reaction. Journal of Clinical Microbiology 1992, 30: 1646–1653.
14. Schempp C, Bocklage H, Lange R, Kölmel HW, Orfanos CE, Gollnick H: Further evidence for *Borrelia burgdorferi* infection in morphea and lichen sclerosis et atrophicus confirmed by DNA amplification. Journal of Investigative Dermatology 1993, 100: 717–720.
15. Huppertz H-I, Schmidt H, Karch H: Detection of *Borrelia burgdorferi* by nested polymerase chain reaction in cerebrospinal fluid and urine of children with neuroborreliosis. European Journal of Pediatrics 1993, 152: 414–417.
16. Schmidt BL, Aberer E, Stockenhuber C, Klade H, Breier F, Luger A: Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in the urine and breast milk of patients with Lyme borreliosis. Diagnostic Microbiology and Infectious Disease 1995, 21: 121–128.
17. Lebech AM, Clemmensen O, Hansen K: Comparison of in vitro culture, immunohistochemical staining, and PCR for detection of *Borrelia burgdorferi* in tissue from experimentally infected animals. Journal of Clinical Microbiology 1995, 33: 2328–2333.
18. Picken RN: Polymerase chain reaction primers and probes derived from flagellin gene sequences for specific detection of the agents of Lyme disease and North American relapsing fever. Journal of Clinical Microbiology 1992, 30: 99–114.
19. Barbour AG: Isolation and cultivation of Lyme disease spirochetes. Yale Journal of Biological Medicine 1984, 57: 521–525.
20. Anderson JF, Magnarelli LA, LeFebvre RB, Andreadis TG, McAninch JB, Perng GC, Johnson RC: Antigenically variable *Borrelia burgdorferi* isolated from cottontail rabbits and *Ixodes dentatus* in rural and urban areas. Journal of Clinical Microbiology 1989, 27: 13–20.
21. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, van der Noordaa J: Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 1990, 28: 495–503.
22. Gaßman GS, Kramer M, Göbel UB, Wallich R: Nucleotide sequence of a gene encoding the *Borrelia burgdorferi* flagellin. Nucleic Acids Research 1989, 17: 3590.
23. Jauris-Heipke S, Fuchs R, Motz M, Preac-Mursic V, Schwab E, Soutschek E, Will G, Wilske B: Genetic heterogeneity of the genes coding for the outer surface protein C (OspC) and the flagellin of *Borrelia burgdorferi*. Medical Microbiology and Immunology 1993, 182: 37–50.
24. Wieslander L: A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Analytical Biochemistry 1979, 98: 305–309.
25. Strle F, Cheng Y, Nelson JA, Picken MM, Bouseman JK, Picken RN: Infection rate of *Ixodes ricinus* ticks with *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto. European Journal of Clinical Microbiology & Infectious Diseases 1995, 14: 994–1001.
26. Picken RN, Cheng Y, Strle F, Cimperman J, Maraspin V, Lotric-Furlan S, Ruzic-Sabljic E, Han D, Nelson JA, Picken MM, Trenholme GM: Molecular characterization of *Borrelia burgdorferi* sensu lato from Slovenia revealing significant differences between tick and human isolates. European Journal of Clinical Microbiology & Infectious Diseases 1996, 15: 313–323.
27. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 1975, 98: 503–517.
28. Guy EC, Stanek G: Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. Journal of Clinical Pathology 1991, 44: 610–611.
29. Melchers WJG, Meis JF, Rosa PA, Claas ECJ, Nohlmans L, Koopman R, Horrevorts A, Galama J: Amplification of *Borrelia burgdorferi* DNA in skin biopsies from patients with Lyme disease. Journal of Clinical Microbiology 1991, 29: 2401–2406.

30. Keller TL, Halperin JJ, Whitman M: PCR detection of *Borrelia burgdorferi* DNA in cerebrospinal fluid of Lyme neuroborreliosis patients. Neurology 1992, 42: 32–42.
31. Williams WV, Callegari P, Freundlich B, Keenan G, Eldridge D, Shin H, Kreitman M, McCallus D, Weiner DB: Molecular diagnosis of *Borrelia burgdorferi* infection (Lyme disease). DNA and Cell Biology 1992, 11: 207–213.
32. Pachner AR, Delaney E: The polymerase chain reaction in the diagnosis of Lyme neuroborreliosis. Annals of Neurology 1993, 34: 544–550.
33. Liebling MR, Nishio MJ, Rodriguez A, Sigal LH, Jin T, Louie JS: The polymerase chain reaction for the detection of *Borrelia burgdorferi* in human body fluids. Arthritis and Rheumatism 1993, 36: 665–675.
34. Wienecke R, Neubert U, Volkenandt M: Molecular detection of *Borrelia burgdorferi* in formalin-fixed, paraffin-embedded lesions of Lyme disease. Journal of Cutaneous Pathology 1993, 20: 385–388.
35. Wienecke R, Zöchling N, Neubert U, Schlüpen E-M, Meurer M, Volkenandt M: Molecular subtyping of *Borrelia burgdorferi* in erythema migrans and acrodermatitis chronica atrophicans. Journal of Investigative Dermatology 1994, 103: 19–22.
36. Moter SE, Hofmann H, Wallich R, Simon MM, Kramer MD: Detection of *Borrelia burgdorferi* sensu lato in lesional skin of patients with erythema migrans and acrodermatitis chronica atrophicans by ospA-specific PCR. Journal of Clinical Microbiology 1994, 32: 2980–2988.
37. von Stedingk LV, Olsson I, Hanson HS, Åsbrink E, Hovmark A: Polymerase chain reaction for detection of *Borrelia burgdorferi* DNA in skin lesions of early and late Lyme borreliosis. European Journal of Clinical Microbiology & Infectious Diseases 1995, 14: 1–5.
38. Christen HJ, Eiffert H, Ohlenbusch A, Hanefeld F: Evaluation of the polymerase chain reaction for the detection of *Borrelia burgdorferi* in cerebrospinal fluid of children with acute peripheral facial palsy. European Journal of Pediatrics 1995, 154: 374–377.