JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1991, p. 731–737
0095-1137/91/040731-07$02.00/0

Vol. 29, No. 4

Comparison of In Vitro Culture and Polymerase Chain Reaction for Detection of *Borrelia burgdorferi* in Tissue from Experimentally Infected Animals

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Received 5 October 1990/Accepted 8 January 1991

A polymerase chain reaction (PCR) was developed for identification of *Borrelia burgdorferi* in biological specimens. The diagnostic efficiency was compared with that of in vitro culture. A primer set specifying a 791-bp DNA fragment of the *B. burgdorferi* B31 flagellin gene was used. Amplified DNA sequences were analyzed by agarose gel electrophoresis, and the identity of amplified DNA was confirmed by restriction enzyme cleavage and Southern blot hybridization with a ^{32}P-labeled probe. By using purified *B. burgdorferi* DNA, the detection limit of the assay was approximately 0.002 pg of DNA, corresponding to one copy of the *B. burgdorferi* genome. By using in vitro-cultivated *B. burgdorferi* without prior DNA purification as the template DNA, 2 to 20 organisms could be detected. A 791-bp DNA fragment was amplified from all of 18 different *B. burgdorferi* strains tested, as well as from *Borrelia hermsii* and *Borrelia anserina* but not from *Treponema pallidum*. The efficacy of the PCR assay was evaluated on spleen, renal, and urinary bladder tissue specimens from eight experimentally infected gerbils. Specimens from the same organs were cultured in BSK medium in parallel. Of 24 organs, 21 (88%) were PCR positive and 17 (71%) were culture positive. All culture-positive specimens were also PCR positive. Compared with *B. burgdorferi* cultivation, PCR had at least a comparable diagnostic sensitivity, it was less laborious, and results were available within 1 to 2 days.

Lyme borreliosis is a multisystemic infection caused by the recently discovered spirochete *Borrelia burgdorferi* (7, 9, 40) and is now the most common tick-borne infection in Europe and North America. If left untreated, the infection may spread from an initially localized erythema migrans and may affect the nervous system, heart, joints, and skin (2, 3, 25, 34, 41, 42). Although the clinical presentation of the different entities of this infection is usually quite typical, there is still a need for accurate diagnostic laboratory tests.

Until now, confirmation of the clinical diagnosis has mainly been achieved indirectly by serological assays that measure antibodies against *B. burgdorferi* (14, 40). Although these tests have been improved (12, 20, 21, 26), serological methods still present three main problems: (i) low diagnostic sensitivity because of a late and slowly appearing antibody response (13, 20, 39), (ii) serological cross-reactivity because of the immunogenic proteins of *B. burgdorferi* that contain epitopes found in a broad range of microorganisms (5, 19, 27), and (iii) an inability to distinguish between actual and past disease because of antibody persistence. Most of these limitations could be circumvented by a reliable and practical diagnostic assay for direct demonstration of *B. burgdorferi* in samples from patients. So far, direct detection of *B. burgdorferi* has been achieved by either histological silver staining or immunostaining (8, 15, 16) or in vitro culture (1, 4, 7, 24, 34, 40). Of these direct diagnostic techniques, in vitro culture has been the most effective, but it is an impractical and low-yield procedure. Considering the paucity of spirochetes in clinical specimens, the recent introduction of in vitro amplification of specific DNA sequences by the polymerase chain reaction (PCR) (36) seems to offer a solution. Several sensitive PCR assays for either purified *B. burgdorferi* DNA or in vitro-cultivated spirochetes have recently been reported (28, 30, 33, 35, 43), but so far there is no study evaluating the diagnostic efficacy of using tissues or body fluids from definitely infected humans or animals.

The aim of the present study was (i) to develop a sensitive PCR assay for *B. burgdorferi* in tissue specimens based on selected parts of the *B. burgdorferi* flagellin gene (18) as the target sequence and (ii) to evaluate the diagnostic sensitivity of this PCR assay compared with that of in vitro culture by using tissue from experimentally infected animals.

(This study was presented at the 4th International Conference on Lyme Borreliosis, Stockholm, Sweden, 18 to 21 June 1990.)

MATERIALS AND METHODS

Spirochetal strains and cultivation. The different strains of *Borrelia* used in the study are summarized in Table 1. *B. burgdorferi*, *Borrelia hermsii*, and *Borrelia anserina* were grown in BSK medium (4) at 32°C to a cell density of 10^8 cells per ml, as determined by dark-field microscopy. The cells were harvested by centrifugation at 10,000 × *g* for 30 min and washed three times in phosphate-buffered saline (pH 7.4) containing 5 mM $MgCl_2$. The final pellet was stored at −20°C until use.

Treponema pallidum (Nicols pathogenic strain) was used as the source of *T. pallidum* DNA. *T. pallidum* was grown in rabbit testicles (32) and harvested by centrifugation on a 45 to 19% Urografin gradient (Schering AG, Berlin, Federal Republic of Germany) at 80,000 × *g* for 60 min (22).

Animal model. Eight male gerbils of the strain *Meriones unguiculatus* (38) (weight, 50 to 70 g; Shamrock, United Kingdom) were infected intraperitoneally with 10^8 *B. burgdorferi* DK1 or DK7 in a low passage to obtain a systemic infection. Three noninfected animals served as controls. On

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TABLE 1. Spirochetal strains used in this study

Strain	Source[a]	Supply
DK1	Skin, EM	Statens Seruminstitut, Copenhagen, Denmark
DK2	Skin, ACA	
DK3	Skin, ACA	
DK4	Skin, EM	
DK5	Skin, ACA	
DK6	CSF	
DK7	Skin, ACA	
DK8	Skin, ACA	
DK9	Skin, ACA	
DK21	Skin, EM	
ACA-1	Skin, ACA	Eva Åsbrink, Södersjukhuset, Stockholm, Sweden (3)
F1	Tick	
B31	Tick	A. G. Barbour, Health Science Center, San Antonio, Tex.
245	Blood	A. C. Steere, Tufts New England Medical Center, Boston, Mass. (40)
272	Skin	
297	CSF	
P/Ko	Skin, EM	V. Preac-Mursic, Max von Pettenkofer Institute, Munich, Federal Republic of Germany
P/Tm	Skin, ACA	
P/Bi	CSF	
Borrelia hermsii		K. Hovind-Hougen, National Veterinary Laboratory, Copenhagen, Denmark
Borrelia anserina		

[a] EM, Erythema migrans; ACA, acrodermatitis chronica atrophicans; CSF, cerebrospinal fluid.

day 24 after infection, the animals were sacrificed; and kidney, spleen, and urinary bladder tissues were removed. Three specimens from every organ were obtained for culture of *B. burgdorferi*. Cultures were initiated in BSK medium by inoculation of a 1- to 2-mm-thick tissue slice without further preparation. The remaining tissue was frozen immediately at −80°C in 0.9% NaCl until tissue preparation for PCR. The organ cultures were incubated at 32°C and examined by dark-field microscopy for growth of spirochetes once a week for 5 weeks.

Purification of spirochetal DNA. Total DNA from the different *Borrelia* strains and *T. pallidum* was, with minor modifications, extracted by previously described protocols (19, 22).

Thawed spirochetes were suspended in 500 μl of SET buffer (25% sucrose, 50 mM EDTA, 50 mM Tris hydrochloride [pH 7.5]). Lysozyme (5 mg/ml), 0.5% (wt/vol) sodium dodecyl sulfate (SDS), DNase-free RNase (0.1 mg/ml), and proteinase K (0.3 mg/ml) were added sequentially. The sample was incubated at 37°C for 1.5 h with gentle shaking every 15 mins. DNA was extracted with three successive phenol extractions and one ether extraction. After adjusting the NaCl concentration to 0.1 M, DNA was precipitated with 2 volumes of 99% (vol/vol) ethanol for 1 h at −20°C and pelleted by centrifugation for 30 min at 30,000 × *g*. The DNA pellet was washed with cold 70% (vol/vol) ethanol, air dried for 1 h, and resuspended in 200 μl of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 7.0]).

DNA was extracted from the *Borrelia* strains listed in Table 1 and from *T. pallidum*. DNA concentrations were estimated after electrophoresis in 0.8% agarose (Sea-Kem FMC, Rockland, Maine)–1× TBE gels (TBE is 0.089 M Tris buffer, 0.089 M borate, and 0.0028 M EDTA) by using standard electrophoresis conditions (29) by comparing the intensity of the ethidium bromide-stained DNA bands with DNA standards of known concentrations (*Hin*dIII digest of bacteriophage lambda DNA).

Sample preparation for PCR from in vitro-cultivated *B. burgdorferi* DK1. A late-logarithmic-phase culture of *B. burgdorferi* DK1 was pelleted and resuspended in phosphate-buffered saline (pH 7.4) to a final cell density of 10^6 *B. burgdorferi* per 10 μl, as determined by dark-field microscopy. Serial dilutions were subsequently made in phosphate-buffered saline to obtain samples with a cell density ranging from 10^5 to 2 *B. burgdorferi* per 10 μl. The samples were heated to 100°C for 10 min in the presence of 1% (vol/vol) Triton X-100 and subsequently chilled on ice. An aliquot of 10 μl was used as template DNA.

Sample preparation for PCR of DNA from gerbil tissue. Total DNA from gerbil tissue was prepared by a standard procedure (11). Approximately 10 mg of tissue was frozen, ground with a pestle (Micropistille; Eppendorf Nethler-Hinz GmbH, Hamburg, Federal Republic of Germany), and suspended in 250 μl of digestion buffer (100 mM NaCl, 10 mM Tris hydrochloride [pH 8.0], 25 mM EDTA [pH 8.0], 0.5% SDS, 0.1 mg of proteinase K per ml). This suspension was incubated in a thermo-mixer (no. 5436; Eppendorf) at 50°C overnight. DNA was extracted with a phenol-chloroform-isoamyl alcohol extraction. The NaCl concentration was adjusted to 0.2 M, and DNA was precipitated by the addition of 2 volumes of 99% (vol/vol) ethanol. The pellet was rinsed with 70% ethanol, air dried, and resuspended in 100 μl of TE buffer. The DNA concentration was estimated, and 100 ng of the preparation was used as template DNA.

In order to optimize the PCR assay for analysis of organ specimens from experimentally infected animals, a controlled number of in vitro-cultivated *B. burgdorferi* DK1, ranging from 10^4 to 10 spirochetes, was added to 10 mg of renal tissue. The DNA was then extracted as described above.

PCR. Oligonucleotide primers were designed based on the nucleotide sequence of the gene encoding the *B. burgdorferi* flagellin protein of strain B31 (18). In order to develop a PCR assay which could discriminate between *B. burgdorferi* and *T. pallidum*, two primer sets were synthesized that were complementary to nonhomologous areas, according to the deduced amino acid sequence of the *B. burgdorferi* 41-kDa flagellin protein and of the Fla B2 subunit from the *T. pallidum* flagellum (18, 31). Primer set I was 5′-ATT AAC GCT GCT AAT CTT AGT-3′ from bases 52 to 72 (primer F1) and 5′-GTA CTA TTC TTT ATA GAT TC-3′ from bases 823 to 842 (primer F3). Primer set II was 5′-CAA AAT GTA AGA ACA GCT GAA-3′ (primer F2) from bases 427 to 447 and primer F3 (Fig. 1).

The primer sets were constructed in such a way that a specific restriction site cut by the enzyme *Ssp*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was positioned asymmetrically in the sequence that was to be amplified (Fig. 1).

PCR was performed in a reaction volume of 50 μl containing 2.5 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.), 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 0.01% gelatin, 3 mM $MgCl_2$, and 250 mM (each) deoxynucleotide triphosphates (dATP, dCTP, dTTP, and dGTP). When purified *B. burgdorferi* DNA or a cell lysate of in vitro-cultivated *B. burgdorferi* was used as the template, a primer concentration of 0.2 mM was used. When purified DNA from gerbil organ tissues was used as the template, optimal amplification needed a higher primer concentration (0.8 mM). The PCR mixture was overlaid with 40 μl of mineral oil (M-3516; Sigma, St. Louis, Mo.). All

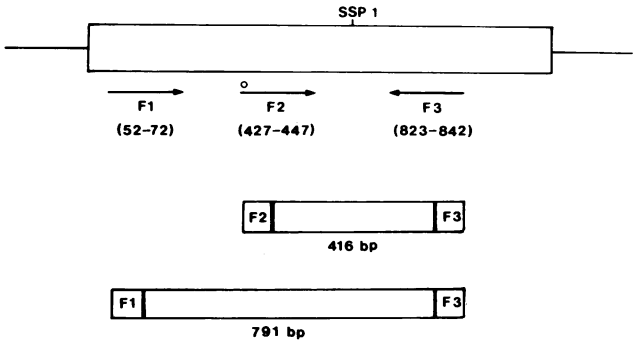

FIG. 1. Schematic map of the *B. burgdorferi* flagellin gene (18) showing the base position (in parentheses) of the oligonucleotide primers used in the PCRs. The orientations of primers are shown with arrows pointing in the 5′ to 3′ direction. The *Ssp*I restriction site at base 564 is indicated. The amplification products expected from the primer sets are shown below.

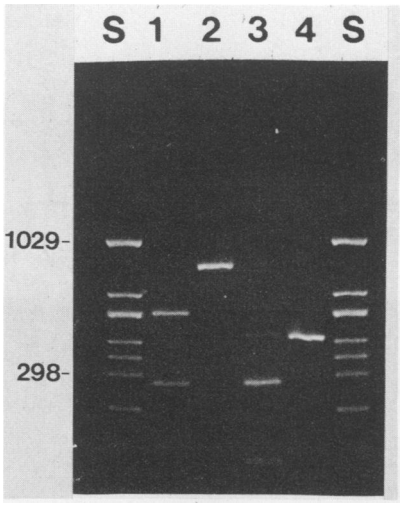

FIG. 2. Amplified fragments of the *B. burgdorferi* flagellin gene and fragments achieved by cleavage with the restriction enzyme *Ssp*I. Primer set I yielded a fragment of 791 bp (lane 2) and, after *Ssp*I cleavage, fragments of 513 and 278 bp (lane 1). For primer set II a fragment of 416 bp (lane 4) was amplified, and fragments of 138 and 278 bp were seen after *Ssp*I digestion (lane 3). The fragments were detected by ethidium bromide staining after agarose gel electrophoresis. *Hin*dIII-*Hin*fI-cleaved pBR322 was used as a DNA size marker (in base pairs) (lane S).

reactions were performed in a thermal cycler (Techne PHC-1; Techne Ltd., Cambridge, United Kingdom). By using purified *B. burgdorferi* DNA or a cell lysate of in vitro-cultivated *B. burgdorferi* as the template, PCR conditions were denaturation at 94°C for 1 min, annealing at 41°C for 30 s, and extension at 66°C for 30 s, for 35 cycles. After the final cycle the temperature was maintained at 66°C for 5 min to complete the extension. For optimal PCR on total DNA extracted from gerbil tissue, conditions were denaturation at 94°C for 1 min, annealing at 39°C for 1 min, and extension at 66°C for 2 min, for 40 cycles. After the final cycle the temperature was also maintained in this case at 66°C for 5 min. The PCRs were analyzed for amplified products by agarose gel electrophoresis (0.8%).

To reduce the risk of contamination, analysis of amplification products was carried out in a separate area from where the PCRs were set up. Pipetting for sample and reagent preparation was carried out with pipettes dedicated for that purpose only. Negative controls (distilled water added to the reaction mixture) were included in every PCR run.

Restriction enzyme analysis of amplification products. After amplification, 10 μl of the PCR mixtures was incubated with *Ssp*I. Digests were then analyzed by agarose gel electrophoresis (1.5%).

Southern blot hybridizations. Southern blots were performed from gels to nitrocellulose filters (BA 83; Schleicher & Schuell, Dassel, Federal Republic of Germany). Prior to transfer, gels were placed in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 60 min, washed twice with distilled water, and finally left in neutralization buffer (1 M Tris hydrochloride [pH 8.0], 1.5 M NaCl) for 60 min. Transfer of DNA to a nitrocellulose filter was performed with a pressure blotter (POSIBLOT; Stratagene, San Diego, Calif.). The oligonucleotide probe F2 was end labeled with [α-^{32}P]dCTP by using a terminal labeling kit (ENZO Diagnostics, Inc., New York, N.Y.). Filters were prehybridized at 42°C for 30 min in a hybridization buffer (0.5 M natrium phosphate buffer [pH 7.2], 1 mM EDTA, 7% SDS) supplied with 100 μg of denatured salmon sperm DNA per ml. Hybridization was performed overnight in hybridization buffer containing the radioactive probe. The filters were then washed three times at 42°C in 100 ml of 0.04 M natrium phosphate buffer (pH 7.2) containing 1% SDS for 15 min and once in 0.02 M natrium phosphate buffer (pH 7.2) containing 0.5% SDS for 15 min. Filters were autoradiographed for 12 to 15 h at −80°C by using one intensifying screen (Lightning Plus J J 300023; Dupont Cronex, Sigma).

RESULTS

When purified DNA of *B. burgdorferi* DK1 was used as the template, PCR yielded a single amplified fragment of the expected size of 791 or 416 bp by using primer set I or II, respectively. The identities of the amplified DNA fragments were confirmed by cleavage with *Ssp*I, which yielded the predicted bands of 513 and 278 bp for primer set I and 278 and 138 bp for primer set II (Fig. 2). The authenticity of the amplification products of primer set I was confirmed by Southern blotting. The PCR-amplified fragment from *B. burgdorferi* DNA hybridized to probe F2, which was from the central part of the target sequence.

To determine the sensitivity of the PCR assay, amplification was performed on serially diluted, purified *B. burgdorferi* DNA ranging from 100 ng to 0.001 pg. When primer set I was used, as little as 0.01 pg was amplified to be detectable by agarose gel electrophoresis (Fig. 3A), whereas, for unknown reasons, primer set II had a detection limit of only 100 pg. The analytical sensitivity obtained with primer set I could be further increased to 0.002 pg when the amplified fragments were detected with ^{32}P-labeled probe F2 in a Southern blot (Fig. 3B). In the following, only results obtained with primer set I are reported.

Purified DNAs from the 18 different *B. burgdorferi* strains listed in Table 1 were subjected to PCR. From all strains an ~791-bp DNA fragment could be amplified, as illustrated in Fig. 4 for 10 strains. The taxonomic specificity of the PCR assay was evaluated by using purified DNA from *T. pallidum*, *B. hermsii*, one of the causative agents of relapsing fever, as well as *B. anserina*, an avian pathogen (Fig. 3A). No amplification products of 791 bp could be detected even when 100 ng of purified *T. pallidum* DNA was used as a

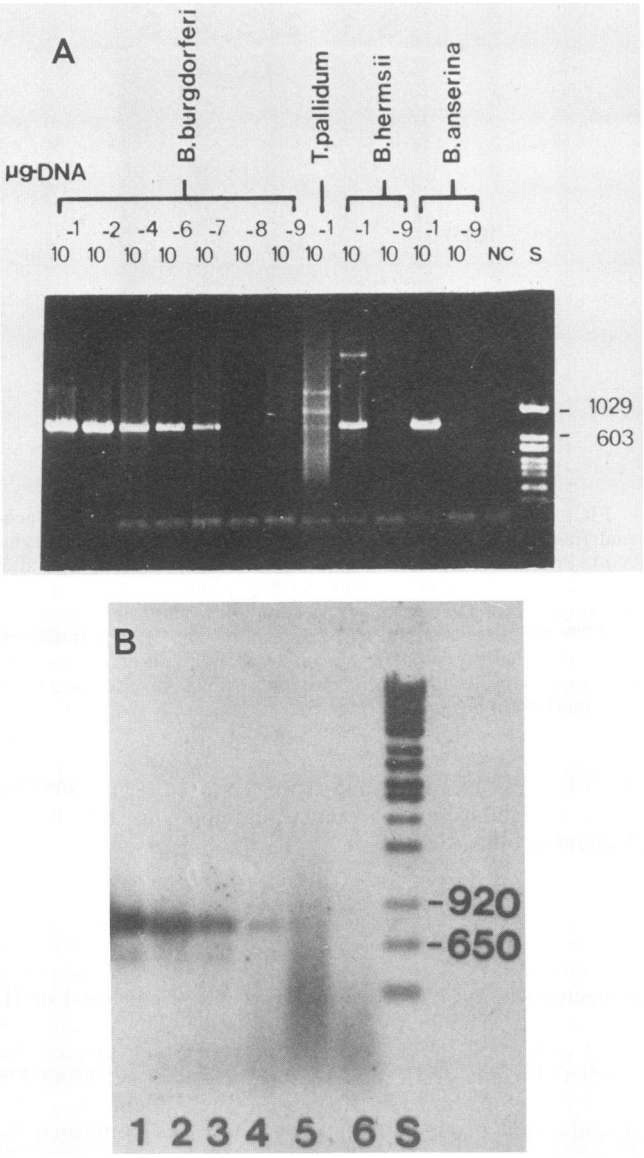

FIG. 3. (A) PCR amplification of *B. burgdorferi* DK1, *T. pallidum*, *B. hermsii*, and *B. anserina* sequences demonstrated by agarose gel electrophoresis after ethidium bromide staining. Sources and amounts (in micrograms) of template DNAs are indicated. NC, Negative control. pBR322 cleaved with *Hin*dIII-*Hin*fI was included as a DNA size marker (in base pairs) (lane S). (B) Southern blot hybridization of PCR amplification of *B. burgdorferi* DK1. A total of 10^{-6} µg (lane 1), 10^{-7} µg (lane 2), 10^{-8} µg (lane 3), 10^{-9} µg (lane 4), and 10^{-10} µg (lane 5) of DNA served as the templates. Lane 6, Negative control. The amplification products were identified by the ^{32}P-end-labeled oligonucleotide F2 (Fig. 1). Lane S, DNA size markers (in base pairs) (^{35}S-labeled DNA size markers; Amersham International plc, Buckinghamshire, United Kingdom).

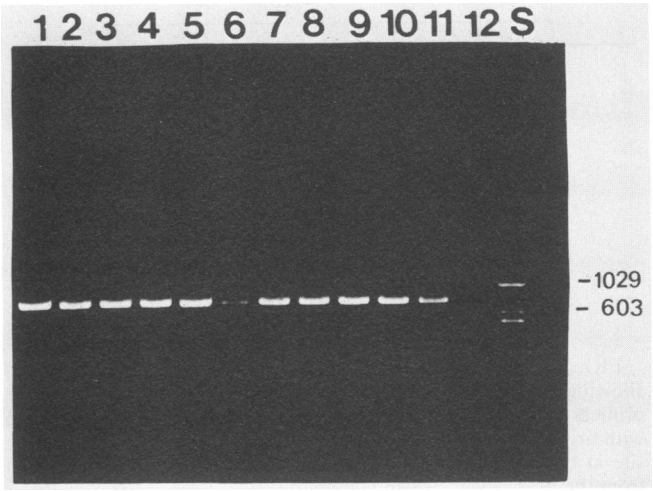

FIG. 4. Agarose gel electrophoresis of PCR products of 10 different *B. burgdorferi* strains. Lane 1, DK1; lane 2, DK2; lane 3, DK3; lane 4, DK4; lane 5, DK5; lane 6, DK6; lane 7, DK7; lane 8, DK9; lane 9, ACA-1; lane 10, B31; lane 11, F1; lane 12, negative control. The remaining eight *B. burgdorferi* strains tested (Table 1) all gave rise to a similar 791-bp fragment. pBR322 cleaved with *Hin*dIII-*Hin*fI was used as a DNA size marker in (in base pairs (lane S).

template. Several faint fragments were produced, including a fragment of 750 bp. However, none of them hybridized to the F2 probe in a Southern blot. By using 100 ng of purified DNA from *B. hermsii* and *B. anserina* as template DNA, amplified fragments with sizes equivalent to those of *B. burgdorferi* appeared; these were cleaved with *Ssp*I and hybridized to the F2 probe in a Southern blot. However, the sensitivity of the PCR assay seemed to be lower, since 0.01 pg of purified *B. hermsii* DNA was not detectable.

The sensitivity of the PCR assay in terms of the minimum number of spirochetes which could be detected was estimated by using samples containing a known number of in vitro-cultivated *B. burgdorferi* DK1. These samples were obtained by a 10-fold dilution series of a spirochetal solution containing 10^6 *B. burgdorferi* per 10 µl. A specific amplification could be achieved from a dilution which theoretically contained 2.5 spirochetes per 10 µl (Fig. 5). Since the number of target molecules in such a low concentration follows a Poisson distribution, we attempted to assess the reproducibility of the assay sensitivity using six different 10-fold dilution series made from the same pool of spirochetes. This experiment showed that even a solution which theoretically contained 10 spirochetes per 10 µl was only PCR positive four of six times. Therefore, it seems reasonable to specify the assay sensitivity as 2 to 20 spirochetes.

PCR analysis was also performed on gerbil tissue to which a controlled number of in vitro-cultivated *B. burgdorferi* cells was added. Under these conditions the assay could detect less than 10 spirochetes. Compared with amplification from purified *B. burgdorferi* DNA and in vitro-cultivated, boiled *B. burgdorferi*, amplification of gerbil tissue supplied with a controlled number of spirochetes needed a fourfold increase in primer concentration and extended annealing and extension time for optimal amplification as described above.

Renal, spleen, and urinary bladder tissue from eight experimentally infected gerbils (total of 24 organ specimens) and three noninfected animals (total of 9 organ specimens) were tested for the presence of *B. burgdorferi* by in vitro culture and PCR. On the basis of culture results, all eight gerbils had a systemic infection with *B. burgdorferi*, since at least two organs were culture positive. Of the 24 organ specimens, 17 (71%) were culture positive. All nine specimens from noninfected animals were culture negative. When PCR analysis was used, 21 of 24 (88%) organs from the eight

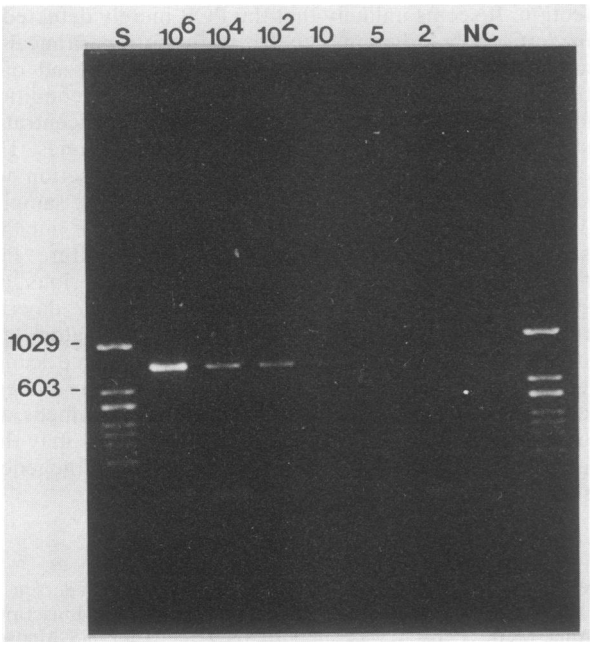

FIG. 5. Sensitivity of the PCR in terms of the number of in vitro-cultivated spirochetes detected in a reaction. The number of spirochetes per 10 μl which was added to the PCR mixture is indicated on top of the lanes in the agarose gel. The amplification products of two spirochetes per 10 μl was only visible on the original photograph. NC, Negative control. pBR322 cleaved with *Hin*dIII-*Hin*fI was used as a DNA size marker (in base pairs) (lane S).

infected animals revealed a distinct band at 791 bp by agarose gel electrophoresis (Fig. 6A). The identities of the amplified fragments were further established by Southern blotting with the ^{32}P-labeled F2 probe (Fig. 6B). None of the specimens from the noninfected gerbils was PCR positive. The results obtained by culture and PCR are compared and summarized in Table 2. All 17 culture-positive specimens were also positive by PCR. A slightly increased sensitivity of the PCR assay is indicated, since four culture-negative samples were positive by PCR.

TABLE 2. Direct demonstration of *B. burgdorferi* in 24 organs of eight experimentally infected gerbils by culture and PCR

PCR result	No. of organs with the following culture result:	
	Positive	Negative
Positive	17	4
Negative	0	3

DISCUSSION

We developed a highly sensitive PCR assay for the detection of *B. burgdorferi* in tissue specimens from experimentally infected animals. Compared with in vitro culture analysis, the PCR assay had a slightly higher sensitivity and was less laborious. Furthermore, results were available within 2 days, whereas culture may take 2 to 8 weeks. Although culture is a low-yield procedure, it has so far been the most effective method for direct detection of *B. burgdorferi* in biological specimens. Because of the paucity of organisms, specific immunohistological staining of biopsy sections from pathological lesions as well as antigen detection in body fluids have not been sufficiently sensitive for diagnostic purposes (23, 41). Even specific DNA probes could not lower the detection limit to less than 10^4 in vitro-cultivated spirochetes (37). Therefore, in vitro amplification of *B. burgdorferi* DNA sequences by PCR became an obvious approach.

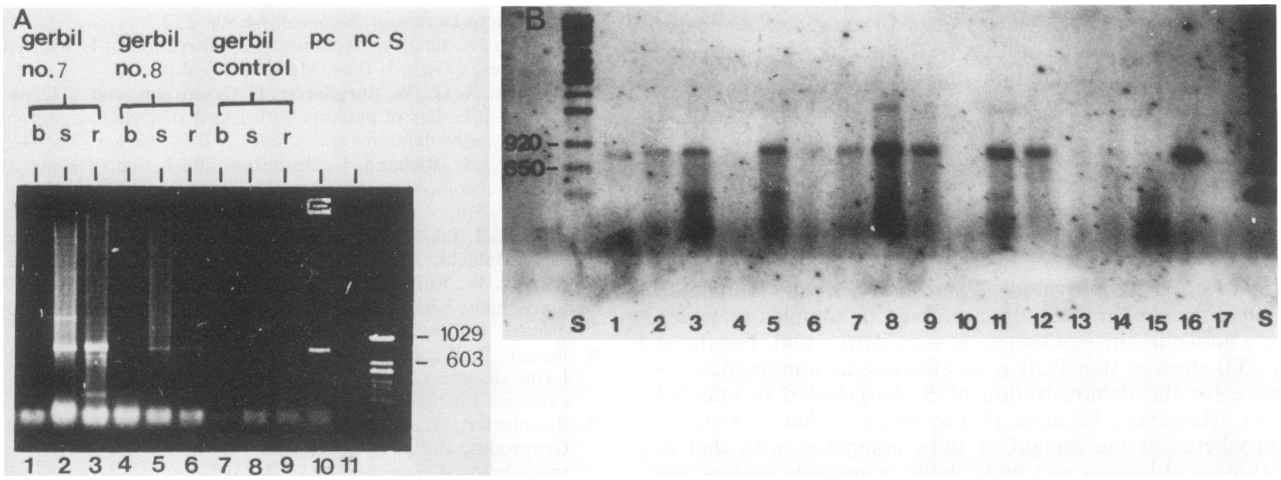

FIG. 6. (A) An agarose gel showing representative PCR results by using tissue from experimentally infected animals. Template DNA was 100 ng of purified DNA from renal (r), spleen (s), and urinary bladder (b) tissue from experimentally infected animals (gerbils 7 and 8; lanes 1 to 6) and from a noninfected animal (lanes 7 to 9). In vitro-cultivated *B. burgdorferi* (10^4) served as a positive control (pc) (lane 10). NC, Negative control (lane 11). pBR322 cleaved with *Hin*dIII-*Hin*fI was used as a DNA size marker (in base pairs) (lane S). (B) Southern blot hybridization showing representative PCR results with tissue from experimentally infected animals. Template DNA was 100 ng of DNA purified from renal, spleen, and urinary bladder tissues from experimentally infected animals (gerbil 1, lanes 1 to 3; gerbil 2, lanes 4 to 6; gerbil 3, lanes 7 to 9; gerbil 4, lanes 10 to 12) and from a noninfected animal (lanes 13 to 15). In vitro-cultivated *B. burgdorferi* (10^4) served as a positive control (lane 16). NC, Negative control (lane 17). The amplification products were identified with the ^{32}P-end-labeled oligonucleotide F2 (Fig. 1). Lane S, DNA size marker (in base pairs) (^{35}S-labeled DNA size markers; Amersham International plc).

Within the past year, five reports on the application of PCR for *B. burgdorferi* detection have been published (28, 30, 33, 35, 43). As target DNA, three of them used parts of the *ospA* gene (28, 30, 33), one used a randomly cloned *B. burgdorferi* sequence selected for its lack of cross-reactivity with other *Borrelia* species (35), and one used, as we did, a part of the flagellin gene. A detection limit of 0.002 to 1.0 pg of purified *B. burgdorferi* DNA was found in all studies. If it is assumed that the *B. burgdorferi* genome is approximately 1,000 bp (6, 17), the sensitivity corresponds to a DNA content of about 2 to 100 spirochetes.

The question of which target DNA sequence should be chosen for optimal diagnostic sensitivity and specificity has not been answered. Because of variations in the DNA sequences of the *ospA* genes of different *B. burgdorferi* isolates, primers based on the *ospA* sequence do not detect every *B. burgdorferi* strain (30, 33), but the reactivity seems to be specific for *B. burgdorferi* (28, 30). The randomly cloned *B. burgdorferi* DNA sequence used by Rosa and Schwan (35) was specific but may not have been sufficient for identification of every *B. burgdorferi* strain, since it did not react with 1 of the 18 isolates tested.

The flagellin gene is extremely conserved among different isolates of *B. burgdorferi*, with there being only four nucleotide differences within the coding region between the U.S. type strain B31 and a European isolate (18). Therefore, we assumed that flagellin gene-derived primers should be able to amplify DNA from all *B. burgdorferi* strains. Being aware of the closely related DNA sequences that encode the flagellins of *B. burgdorferi* (18) and *T. pallidum* (10, 31), we placed the oligonucleotide primers F1 and F2 in nonhomologous areas. Our PCR assay was able to amplify DNA from all 18 different *B. burgdorferi* strains tested, and as expected, there was no cross-reactivity even with high amounts of *T. pallidum* DNA. On the other hand, the assay did not discriminate *B. burgdorferi* from other *Borrelia* species such as *B. hermsii*. However, from a medical viewpoint, this cross-reactivity is acceptable, as long as it is known, especially since (i) relapsing fever agents cause disease that is clinically distinct from Lyme borreliosis and (ii) clinical cases of relapsing fever do not occur in those areas primarily endemic for Lyme borreliosis, such as Europe and the northeastern part of North America. This limitation in taxonomic specificity may possibly be solved by using the recently reported DNA sequence data of the *B. hermsii* flagellin gene. Primers complementary only to areas of the *B. burgdorferi* flagellin gene and not to the *B. hermsii* flagellin gene may selectively amplify purified *B. burgdorferi* DNA (34a).

Most previous studies that evaluated the performance of PCR for *B. burgdorferi* detection used purified DNA as the target, in vitro-cultivated *B. burgdorferi*, and simulated clinical specimens i.e., urine and serum samples to which were added in vitro-cultivated *B. burgdorferi* (28). Persing et al. (33) showed that PCR is as effective as immunofluorescence for the demonstration of *B. burgdorferi* in infected ticks. However, because of the much higher density of spirochetes in the midgut of ticks compared with that in pathological lesions and body fluids of patients with active Lyme borreliosis, the conditions are not comparable. We reported here a PCR assay for demonstration of *B. burgdorferi* in tissue specimens from infected mammals. The assay was optimized and evaluated by using tissue specimens from experimentally infected animals, which, we assumed, are almost identical to human specimens. The high accordance with culture results indicates that the PCR most likely detected organisms that multiplied because of an established infection. It seems unlikely that the PCR merely detected *B. burgdorferi* DNA disseminated from the intraperitoneal inoculum of spirochetes. When animal tissue instead of in vitro-cultivated *B. burgdorferi* was used, PCR conditions had to be changed by increasing the primer concentration and by extending the annealing and extension times. Furthermore, purification of DNA by phenol extraction was necessary to yield reproducible results from tissue samples.

We conclude that the diagnostic performance of our PCR assay was at least equivalent to that of in vitro culture. PCR tended to be slightly more sensitive, was less laborious, and was faster since results were available within 2 days. A further advantage of the PCR assay is that confirmatory tests can be repeated several times on the same tissue sample even after storage for a long time. The technique is being adapted for analysis of human skin biopsy specimens and samples of blood, cerebrospinal, and joint fluid. It may then become a useful tool in medical diagnosis and pathogenetic research.

ACKNOWLEDGMENTS

We thank Helle Fjordvang and Dorthe Søeborg Pedersen for technical assistance and Karin Larsen for typing the manuscript.

Anne-Mette Lebech was supported by the Thorvald Madsens Legat and Dakopatts. Klaus Hansen was supported by grant 12-9059 from the Danish Medical Research Council. Peter Hindersson was supported by the Danish Medical Research Council, Gigtforeningen, and NOVO's Fond. The work was supported by the Research Center for Medical Biotechnology under the Danish Biotechnological Research and Development Program.

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