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Comparison of In Vitro Culture, Immunohistochemical Staining, and PCR for Detection of *Borrelia burgdorferi* in Tissue from Experimentally Infected Animals

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An avidin-biotin-amplified immunophosphatase staining method with a purified polyclonal rabbit anti-*Borrelia burgdorferi* hyperimmune serum was developed for identification of *B. burgdorferi* in tissue specimens. The diagnostic efficacy was compared with those of in vitro culture and PCR with fresh and fixed, paraffin-embedded tissues. A nested PCR assay was developed for identification of a 276-bp fragment of the *B. burgdorferi* flagellin gene. The diagnostic sensitivities of the different techniques were evaluated with spleen, renal, and urinary bladder tissues from eight experimentally infected gerbils. A systemic infection was verified by positivity of 23 of 24 (96%) organ cultures. *B. burgdorferi* was visualized immunohistochemically in 9 of 23 (39%) of the specimens. Among these nine specimens, an average of 33% of the 15 sections examined were positive. The spirochetes accumulated in discrete clusters and were associated with focal lymphocytic infiltration. The diagnostic sensitivity obtained by PCR with fixed, paraffin-embedded tissue was 21%, considerably lower than that with fresh tissue (71%). Thus, the reliable demonstration of *B. burgdorferi* by immunohistochemical staining is possible but extremely laborious, and considering the fact that the density of *B. burgdorferi* in human tissue is even lower than that in experimentally infected animals, the method is not useful in a clinical setting. It may, however, still be valuable in pathogenetic research. Detection of *B. burgdorferi* DNA by PCR should be performed with fresh tissue specimens and not with fixed, paraffin-embedded specimens.

Ever since the discovery of *Borrelia burgdorferi*, a main issue has been the development of specific assays for direct detection of the spirochete or components of it in specimens from patients with Lyme borreliosis. In this respect, the fundamental problem seems to be the extremely small number of spirochetes in pathological lesions and body fluids, which explains why so far, even 12 years after the discovery of the spirochete, no reliable routine test for direct detection of the organism exists.

In vitro cultivation is a reliable method for the direct demonstration of *B. burgdorferi* in clinical samples and is still regarded as the "gold standard." However, cultivation of *B. burgdorferi* from clinical specimens is a difficult, time-consuming, and (except with skin specimens) low-yield procedure. A positive culture from skin biopsies in 40 to 70% of patients with erythema migrans (EM) or acrodermatitis chronica atrophicans (ACA) is reported (3, 7), but cultures are rarely positive until 2 to 4 weeks of incubation. In Lyme neuroborreliosis the success rate with cerebrospinal fluid may reach 10% within the first week of disease (20), and successful isolation from blood has only rarely been reported (5, 33).

Histochemical staining with silver has been used to visualize *B. burgdorferi* in different tissues from patients with Lyme borreliosis (6, 10, 12, 19) or in tissue sections from experimentally infected animals (4, 11, 29). However, silver staining methods are not specific. *B. burgdorferi*-specific immunohistochemical staining of skin biopsies from patients with dermatoborreliosis by using either polyclonal or monoclonal antibodies has been reported (1, 27), but for a limited number of patients. The diagnostic sensitivities of silver impregnation and specific immunohistochemical staining techniques for identification of *B. burgdorferi* in tissue sections have been neither systematically evaluated nor compared with in vitro culture or PCR.

Regarding the diagnostic performance of PCR with clinical specimens, the data so far available are variable, and with the exception of use with skin biopsies, the value of PCR as a reliable diagnostic test is not yet clear. In tissue from experimentally infected animals (16, 22, 25) as well as in skin biopsies from patients with EM and ACA (23, 30), PCR has a diagnostic sensitivity comparable to that of culture. PCR with cerebrospinal fluid from patients with defined Lyme neuroborreliosis is positive only in 20 to 25% of cases (2, 21), whereas in a recent but single paper, PCR was reported successfully to detect *B. burgdorferi* in synovial fluid from 75 of 88 patients with Lyme arthritis (24).

So far, all PCR studies with clinical specimens have been performed with fresh, nonfixed tissue. An adaptation of a *B. burgdorferi*-specific PCR for formalin-fixed, paraffin-embedded tissue would be very advantageous, since both histopathological analysis and diagnostic PCR could be performed with the same biopsy. If possible, this would also allow the study of stored tissue blocks.

The aim of the present study was, by the use of in vitro culture-positive tissue specimens from experimentally infected animals, (i) to investigate the diagnostic sensitivity of a *B. burgdorferi*-specific avidin-biotin-amplified immunphosphatase staining method and (ii) to evaluate the diagnostic performance of PCR with both fresh and fixed, paraffin-embedded tissues. The results are expected to predict the efficacies of the different methods as diagnostic tools for human Lyme borreliosis.

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MATERIALS AND METHODS

Animal model. Eight male gerbils of the strain *Merioners unguiculatus* (31) (weight, 50 to 70 g; Shamrock, United Kingdom) were infected intraperitoneally with 10^8 *B. burgdorferi* DK1 (subculture no. 9) organisms in order to obtain a systemic infection. Three noninfected animals served as controls. On day 24 after infection, the animals were sacrificed and the kidney, spleen, and urinary bladder were removed. Specimens from every organ were (i) inoculated in BSK medium (3a) for culture of *B. burgdorferi*, (ii) immediately frozen at −80°C in 0.9% NaCl until tissue preparation for PCR, or (iii) fixed in phosphate-buffered formalin (10%) and embedded in paraffin for immunohistochemical staining and PCR.

Three cultures of each organ (kidney, spleen, and urinary bladder) were initiated in BSK medium by inoculation of a 1- to 2-mm-thick tissue slice without further preparation. The organ cultures were incubated at 32°C and examined by dark-field microscopy for growth of spirochetes once a week for 6 weeks. For immunohistological staining and PCR, the samples were prepared as described below.

Immunohistochemical staining. Gerbil tissue was fixed in phosphate-buffered formalin (10%) (pH 7.0) at room temperature for 16 to 20 h, embedded in paraffin, and sectioned. The paraffin was removed from series of 5-μm sections with xylene, and the sections were rehydrated through a graded ethanol series. The sections were immersed in a 0.1% solution of trypsin (type II, no. T-8128; Sigma) (pH 7.8) at 37°C for 20 min and neutralized by washing with tap water and Tris-buffered saline (pH 7.6). Slides were blocked by immersion in 10% goat serum (code x-907; DAKO, Copenhagen, Denmark) for 10 min to suppress nonspecific immunoglobulin staining and then incubated with the purified rabbit polyspecific antibody to *B. burgdorferi* (BN98) diluted 1:16,000 in Tris-buffered saline at 4°C overnight. (The production of the antibody BN98 has been described previously [15]). The slides were then treated consecutively with (i) biotinylated goat anti-rabbit immunoglobulin (code E432; DAKO) diluted 1:300, (ii) alkaline phosphatase-conjugated streptavidin (code D396; DAKO) diluted 1:100, and (iii) biotinylated alkaline phosphatase (code K391; DAKO) diluted 1:1,000. All reagents were diluted in Tris-buffered saline and incubated for 30 min. Washes were performed after each incubation step. Slides were incubated with the New Fuchsin substrate system (code K698; DAKO), and the color reaction was allowed to develop for 12 to 20 min. Following the staining reaction, the slides were washed thoroughly in tap water, counterstained with Mayer's hematoxylin (code 254; Bie & Berntsen A/S, Rødovre, Denmark), dehydrated, and mounted with coverslips. Controls included were (i) positive controls (human postmortem skin injected with a live *B. burgdorferi* DK1 culture), (ii) negative controls (tissue sections from noninfected control animals), and (iii) tissue sections from *B. burgdorferi*-infected animals for which incubation with the *B. burgdorferi*-specific indicator antibody was omitted.

From each organ a series of at least 15 sections (range, 15 to 42) was examined for the presence of *B. burgdorferi*. An organ specimen was considered positive if at least two consecutive slides contained *B. burgdorferi* antigen. The sections from infected and noninfected animals were coded and the microscopic examination was performed blindly by one of the authors (A.-M.L.).

In addition, three sections of each organ specimen were stained with hematoxylin and eosin for histopathological examination.

Sample preparation for PCR. (i) Formalin-fixed and paraffin-embedded gerbil tissue. Tissue was fixed in phosphate-buffered formalin (10%) (pH 7.0) at room temperature for 16 to 20 hours, dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. The paraffin-embedded tissue blocks were prepared according to a previously described protocol (34) with minor modifications. Briefly, 10 sections of 5 μm were cut from the blocks, excess paraffin was removed, and the tissue sections were transferred to a microcentrifuge tube. The paraffin was removed from the sections with xylene, and the sections were washed with 70% ethanol to remove the organic solvent. The desiccated tissue was then resuspended in 200 μl of digestion buffer (50 mM Tris [pH 8.5], 1 mM EDTA, 0.5% Tween 20) with proteinase K (200 μg/ml) and then incubated at 37°C overnight or at 60°C for 3 h. One hundred fifty microliters of a 5% Chelex-100 resin solution (catalog no. 142-2832; Bio-Rad, Richmond, Calif.) was added to the preparation, which then was heated to 100°C for 10 min, centrifuged at 3,000 × *g* for 1 min, and subsequently chilled on ice. The DNA concentration in the supernatant was estimated by agarose gel electrophoresis, and approximately 50 to 100 ng of the preparation was used as the source of template DNA.

(ii) Fresh gerbil tissue. Total DNA from gerbil tissue was prepared according to a previously described protocol (9). The DNA concentration was estimated by agarose gel electrophoresis, and 100 ng of the preparation was used as template DNA.

PCR. A nested PCR assay was developed. Oligonucleotide primers were based on the nucleotide sequence of the highly conserved flagellin-encoding gene of *B. burgdorferi* (14). To obtain a *B. burgdorferi*-specific amplification, the primers were placed in areas nonhomologous to the nucleotide sequences of the *Borrelia hermsii* (28) and *Treponema pallidum* (8, 26) flagellin genes.

The outer primer set F1-F3 amplified a 791-bp fragment of the flagellin gene, and the inner primer set F6-F8 amplified a 275-bp fragment. The sequences and positions of the primers and the verification probe within the flagellin gene are shown in Table 1. Oligonucleotide primers were synthesized on an Applied Bio-Systems PCR-Mate DNA synthesizer. They were used after ethanol precipitation and resuspension in Tris-EDTA buffer. PCR was performed in a reaction volume of 50 μl containing 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 0.01% gelatin, 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dTTP, and dGTP), and 1 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.). The outer primer set F1-F3 required 3.5 mM $MgCl_2$, and the inner primer set F6-F8 required only 1.5 mM $MgCl_2$. An amount of 10 pmol of each of the primers F1 and F3 was utilized. After an initial denaturation at 94°C for 2 min, the PCR conditions were 35 cycles of denaturation for 94°C for 1 min, annealing at 41°C for 2 min, and extension at 66°C for 3 min. Two microliters of the PCR mixture was then used as template DNA in the second amplification. The inner primer set F6-F8 (50 pmol) was used for another 35 cycles. After an initial denaturation at 94°C for 2 min, the PCR conditions were denaturation for 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min.

TABLE 1. Sequences and positions of oligonucleotide primers for PCR

Primer	Sequence	Coding strand	Location (nt)[a]
F1	ATT AAC GCT GCT AAT CTT AGT	+	52–72
F3	GTA CTA TTC TTT ATA GAT TC	−	823–842
F6	TTC AGG GTC TCA AGC GTC TTG GAC T	+	492–516
F8	GCA TTT TCA ATT TTA GCA AGT GAT G	−	743–767
F7[b]	CTC TGG TGA GGG AGC TCA AAC	+	594–614

[a] Nucleotide (nt) positions are numbered according to the published sequence of the gene for the 41-kDa *B. burgdorferi* flagellin (14).
[b] ^{32}P-labeled oligonucleotide probe.

The PCR mixture was overlaid with 45 μl of mineral oil (M-3516; Sigma). All reactions were performed in a thermal cycler (Techne PH-C-1; Techne Ltd., Cambridge, United Kingdom). The safety conditions to avoid false-positive PCRs due to contamination were as previously specified (21). Each PCR experiment included negative controls in which water replaced template DNA and a positive control which contained 1 ng of purified DNA from *B. burgdorferi* DK1. Reloading of template DNA for amplification with the inner primer set was performed in a third laboratory.

The specificity of the PCR products was confirmed by DNA-DNA hybridization. Southern blotting and slot blotting were performed as described previously (21). The oligodeoxynucleotide F7 was end labeled with [α-^{32}P]dCTP by using a terminus labeling kit (ENZO Diagnostics, Inc., New York, N.Y.). Membranes were hybridized and washed at 45°C and were subsequently autoradiographed.

The analytical sensitivity for PCR detection of purified DNA was established with serially diluted, purified *B. burgdorferi* DK1 DNA. The detection limit obtained was approximately 0.01 pg when the amplified fragments were detected with the radioactively labeled probe F7. The sensitivity of the PCR assay in terms of the minimum number of in vitro-cultivated *B. burgdorferi* cells without prior DNA extraction was determined with spirochetal solutions containing 10^6 to 10 spirochetes per 10 μl. A reproducible amplification was achieved when 10 spirochetes were added to the PCR mixture. These samples were prepared as previously described (22).

The present PCR assay equally amplified DNAs from a panel of 20 different *B. burgdorferi* isolates representing all three genospecies of *B. burgdorferi* sensu lato (21). The inner primer set was responsible for the *B. burgdorferi*-specific amplification (28), since the outer primer set was previously shown to also amplify *B. hermsii* (22).

Statistical analysis. The diagnostic sensitivities of immunohistochemical staining and PCR with either fixed, paraffin-embedded tissue or fresh tissue were compared by McNemar's test. The calculations were performed pairwise by using the total number of organs for each method.

RESULTS

In vitro culture. Renal, spleen, and urinary bladder tissues from eight experimentally infected gerbils (total, 24 organ specimens) and three noninfected gerbils (total, 9 organ specimens) were examined for the presence of *B. burgdorferi* by in vitro culture. Of the 24 organ specimens, 23 (96%) were culture positive (Table 2). All nine specimens from noninfected animals were culture negative.

Immunohistochemical staining. Spirochetal structures stained intensely red in contrast to the hematoxylin-stained background tissue. The connective tissue and muscle fibers stained diffuse light red. Figure 1 shows the immunohisto-

TABLE 2. Demonstration of *B. burgdorferi* in different organs of eight experimentally infected gerbils by culture, immunohistochemical staining, and PCR

Organ	Sensitivity (no. positive/no. tested) of:			
	Culture	Immunohistochemical staining	PCR with:	
			Paraffin-embedded tissue	Fresh tissue
Kidney	8/8	0/7[a]	1/8	5/8
Spleen	7/8	5/8	1/8	5/6[b]
Urinary bladder	8/8	4/8	3/8	ND[c]
Total	23/24 (96%)	9/23 (39%)	5/24 (21%)	10/14 (71%)[d]

[a] One block was accidentally destroyed during preparation.
[b] For two animals no tissue was left over after the three other analyses were performed.
[c] ND, Not done because no tissue was left over after the three other analyses were performed.
[d] $P < 0.05$ versus immunohistochemical staining; $P < 0.01$ versus PCR with paraffin-embedded tissue.

chemical staining of a section from the positive control (human postmortem skin injected with *B. burgdorferi*).

The specificity of the immunostaining assay was controlled by (i) omission of the anti-*B. burgdorferi* indicator antibody (BN98) and (ii) investigation of tissue from noninfected gerbils. In none of these cases was there a staining reaction.

Tissues from the eight experimentally infected gerbils (24 organ specimens) and three noninfected gerbils (9 organ specimens) were immunostained and examined for the presence of *B. burgdorferi*. Of 23 organ specimen (one block was accidentally destroyed during preparation), 9 (39%) stained positive (Table 2). On the basis of the examination of at least 15 sections, spirochetes were found in 11 to 70% (average, 33%) of the sections from a positive organ.

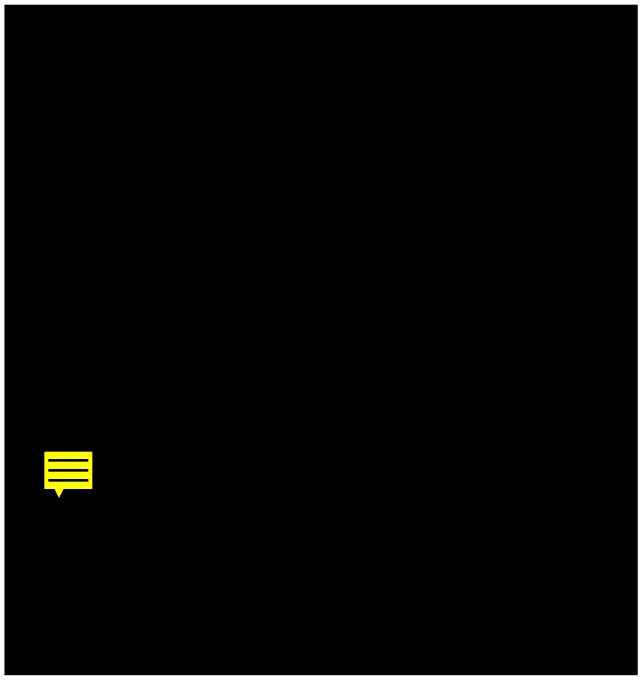

FIG. 1. Biopsy of human skin injected postmortem with *B. burgdorferi* DK1 (positive control). Immunohistochemical staining was performed with a polyspecific anti-*B. burgdorferi* antibody. (Magnification, ×250.)

FIG. 2. Spleen specimen from an experimentally infected gerbil. Immunohistochemical staining was performed with a polyspecific anti-*B. burgdorferi* antibody (BN98). Several single spirochetes associated with a lymphohistiocytic infiltrate are seen. (Magnification, ×250.)

As summarized in Table 2, the presence of spirochetes varied among tissues. No spirochetes were found in renal specimens. In spleen and urinary bladder specimens, the spirochetes occurred in small focal clusters, as usually three or four organisms were observed in one area whereas other areas were completely devoid of organisms.

The presence of spirochetes was associated with an inflammatory cell infiltrate dominated by lymphocytes but with an admixture of eosinophils and plasma cells (Fig. 2). In the spleen, five animals showed a moderate to marked infiltration of lymphocytes surrounding the lymphoid follicles accompanied by giant cells. In the urinary bladder, submucosal infiltrates of lymphocytes was observed in six animals, whereas only in one animal was the muscle layer involved. No histological abnormalities were found in the kidneys.

Detection of *B. burgdorferi* DNA. *B. burgdorferi* DNA was detectable by PCR in 10 of 14 (71%) nonfixed renal and spleen specimens from infected animals. Bladder tissue from all eight animals and spleen tissue from two animals were not examined, because no tissue was left after in vitro culture, immunohistochemical staining, and PCR with fixed, paraffin-embedded tissue were performed. Regarding the paraffin-embedded organ specimens from eight infected animals, *B. burgdorferi* DNA was detectable in only 5 of 24 specimens (21%). The amplified 275-bp fragment was visible by both agarose gel electrophoresis and Southern blotting (Fig. 3). All results obtained by in vitro culture, immunohistochemical staining, and PCR with both fresh and fixed, paraffin-embedded tissues are summarized in Table 2. PCR with fresh tissue was significantly more sensitive than specific immunohistochemical staining and PCR with fixed, paraffin-embedded tissue.

DISCUSSION

In 1983, Berger et al. (6) convincingly demonstrated spirochetes in 4 of 14 skin biopsies from patients with EM by using

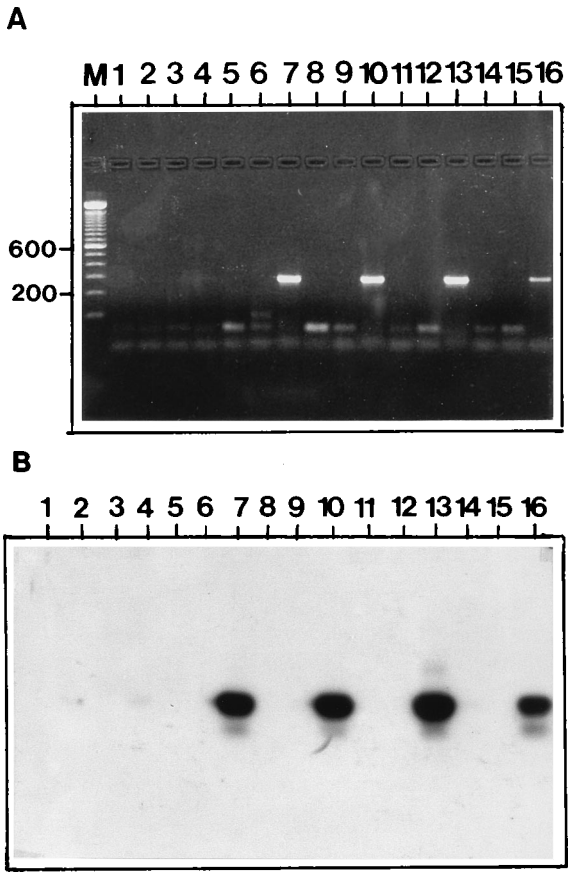

FIG. 3. (A) Agarose gel showing representative PCR results with paraffin-embedded tissue specimens from experimentally infected animals. The template DNAs were DNAs from urinary bladder tissues from noninfected animals (lanes 1 to 6) and from experimentally infected animals (lanes 7 to 14). Lane 15, negative control; lane 16; 1 ng of purified DNA from *B. burgdorferi* DK1 as positive control; lane M, DNA size markers in base pairs. (B) Southern blot hybridization of the gel shown in panel A. The amplification products were identified with the ^{32}P-end-labeled oligonucleotide F7.

a silver staining method. Some authors still use silver staining techniques (10, 12, 19). However, in general, specific immunostaining methods should be preferred because of a higher specificity and no risk of artifacts due to interpretation of argentophilic structures as spirochetes.

Despite this preference, only a few reports on *B. burgdorferi*-specific immunostaining have been published (1, 27). Park et al. (27) demonstrated *B. burgdorferi* in a single skin biopsy from a patient with EM by using a murine monoclonal antibody (H9724) directed against a genus-specific epitope of the *Borrelia* flagellum. On the basis of this single biopsy, they found silver staining to be superior to immunostaining. Another study used a human polyclonal anti-*B. burgdorferi* serum from a patient with ACA as the indicator antibody and reported the successful identification of *B. burgdorferi* in approximately 30% of skin biopsies from patients with EM and ACA (1). However, *B. burgdorferi*-like structures were also identified in patients with other skin diseases usually not attributed to Lyme borreliosis.

Studies that systematically evaluate the diagnostic sensitivity of immunohistochemical staining have so far not been reported. Our present study thus intended, by the use of organs from experimentally infected animals, systematically to investigate two issues: (i) the utility of an avidin-biotin-amplified immunophosphatase staining method with a purified polyclonal rabbit anti-*B. burgdorferi* hyperimmune serum and (ii) the utility of a *B. burgdorferi*-specific PCR with fixed, paraffin-embedded tissue for diagnostic identification of *B. burgdorferi*. Although we were aware that a monoclonal antibody would ensure a higher specificity of the immunostaining method, we used a polyclonal, polyspecific anti-*B. burgdorferi* serum because previous experience with a monospecific anti-*Borrelia* flagellum antibody revealed nonhomogeneous staining of the spirochetes, thus lowering the visual discrimination (27). By use of the immunostaining method, *B. burgdorferi* could be demonstrated in 39% of the specimens, compared with 96% by culture. The visual appearance of the spirochetes was distinct and clear, but their sparsity required a meticulous search. Like in a previous study (4), the spirochetes were located extracellularly, showed a predilection for connective tissue, and were recognized in clusters. Such foci typically contained three or four spirochetes, whereas adjacent areas were totally devoid of spirochetes. In the present study, the density of spirochetes varied significantly between organs. Whereas *B. burgdorferi* was easily visualized in approximately 50% of spleen and urinary bladder specimens, no spirochetes were detected in the kidneys. This was unexpected, since renal infection was documented by culture and PCR. In accordance with the lack of spirochetes, we found no histological abnormalities in the kidneys. Previous studies using silver staining techniques with tissue from experimentally infected hamsters and mice (4, 11) showed spirochetes in both spleen and renal specimens; there was no mention of a difference in density. Those authors reported spirochetes to be located in the renal tubules (11) or proximal to the adventitia of the renal arteries (4). We were unable to reproduce these findings. In a previous study the number of organisms in different organs was found to depend on the duration of the experimental infection, with the highest number of spirochetes seen by 2 weeks (4). This may explain the finding by Preac Mursic et al. (29), who 6 weeks after experimentally infecting gerbils found visible spirochetes only in abdominal lymphnodes and stomach tissue, whereas culture proved a systemic infection, since positive cultures were obtained from skin, heart, and joint tissues but not from renal or spleen tissue. Urinary bladder tissue was not examined by culture. Since the organs used in our study were collected on day 24 after infection, this may explain the lower content of spirochetes. In agreement with other reports (4, 29), despite a systemic infection we found only a slight to moderate inflammatory host reaction, which nevertheless frequently was helpful in identifying the location of spirochetes.

The number of spirochetes in skin biopsies from patients with EM seems to be considerably lower than that in tissue from experimentally infected gerbils (8a). Thus, we assume that the diagnostic sensitivity of the immunohistochemical staining with clinical specimens will be too low to be of any diagnostic value.

Confirming a previous report (22), this study found that PCR with fresh tissue from experimentally infected animals had a diagnostic sensitivity comparable to that of culture. A similar conclusion was drawn by Hofmeister et al. (16) and Pachner et al. (25), although Pachner et al. did not report the precise value obtained for the sensitivity of either PCR or culture. Because the amount of tissue used for culture exceeds that used for PCR and immunohistochemical staining, a direct comparison of the diagnostic efficacies of the methods is not fully justifiable; however, because of the inherent necessity of tissue for cultivating *B. burgdorferi*, this comparison is inevitable. Previous comparisons of in vitro culture and PCR efficacy also could not take this inequality into account. In any case, we

decided to include in vitro culture, since this method is still the most accepted documentation for *B. burgdorferi* infection. In our present study, the amount of bladder tissue unfortunately was not sufficient to perform all four procedures. Thus, we decided to omit PCR with fresh bladder tissue because PCR with this tissue source previously proved to be highly sensitive (22).

Adaptation of PCR for the detection of *B. burgdorferi* in formalin-fixed, paraffin-embedded tissue would be very advantageous. The same skin biopsy could then be used for both histological examination and diagnostic PCR. Furthermore, this would allow the study of stored material retrospectively. So far, the detection of *B. burgdorferi* DNA by PCR in extracts from fixed, paraffin-embedded skin or tissue specimens has not been reported. In the present study, we showed that the diagnostic sensitivity obtained for PCR with fixed, paraffin-embedded specimens was only 21%, compared with 71% when DNA was extracted from fresh tissue. PCR has successfully been used to detect genes from eukaryotic and viral genomic DNAs in formalin-fixed, paraffin-embedded tissue (17, 32). However, fixatives cause DNA degradation, and PCR with DNA extracted from fixed sections is generally less efficient than PCR with DNA prepared from fresh tissue (18). The degradation of DNA depends strongly on the fixative and the fixation time (18). Thus, we chose neutral buffered formalin fixation for less than 24 h because this protocol was reported to be the least destructive (13, 18). Furthermore, and in agreement with results of other studies (13, 34), we observed *Taq* polymerase inhibition by DNA extracted from fixed tissue. This effect was partly overcome by using a nested PCR approach. From a practical point of view, however, the nested PCR is not attractive because of an increased risk of contamination. These factors probably contribute to the very reduced sensitivity obtained by PCR with fixed, paraffin-embedded tissue. Another reason is that the number of spirochetes present in lesions from patients with Lyme borreliosis is very low, which is in contrast to the case for most viral infections, in which the number of viral particles is higher. It might be argued that the observed differences in the sensitivity of PCR with fixed and fresh tissue could be partly attributed to the larger amount of fresh tissue used for DNA extraction. However, since the same amount of extracted DNA was used as template DNA in the two situations, this seems unlikely. Thus, from these results we assume that PCR with paraffin-embedded tissue from human skin biopsies is unlikely to be a useful diagnostic approach.

We conclude that *B. burgdorferi* can be detected in tissue sections from experimentally infected animals by an avidin-biotin-amplified immunophosphatase staining method but that the diagnostic sensitivity is low. The immunohistochemical staining technique is extremely laborious, and considering the fact that the density of *B. burgdorferi* in human tissue is even lower than that in experimentally infected animals, the method is not useful in a clinical setting. It might, however, still be a valuable tool in pathogenetic research. The diagnostic sensitivity obtained by PCR with fixed, paraffin-embedded tissue from experimentally infected animals was low. In contrast to PCR with unfixed skin biopsies, diagnostic PCR with paraffin-embedded tissue from human skin biopsies is unlikely to become a useful diagnostic approach.

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