CLINICAL MICROBIOLOGY REVIEWS, Jan. 1997, p. 185–201 Vol. 10, No. 1
0893-8512/97/$04.00+0

PCR in Laboratory Diagnosis of Human *Borrelia burgdorferi* Infections

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INTRODUCTION

PCR has been employed extensively in the medical and biological sciences since it was formally introduced at the Cold Spring Harbor 51st Symposium on Quantitative Biology (120). PCR for the diagnosis of infectious diseases has been directed primarily toward the detection of pathogens for which conventional diagnostic techniques are either too insensitive or too slow.

Lyme borreliosis (also referred to as Lyme disease) is the most prevalent vector-borne disease in the United States and is endemic in parts of Europe and Asia (167). Current laboratory diagnosis of Lyme disease relies on methods with known limitations. The diagnostic value of antibody assays is unsatisfactory in early disease due to low sensitivity (29, 30), serological cross-reactions (104), and the inability to distinguish between active and inactive infection due to antibody persistence after therapy (42). The pathogen can also be detected by culture; however, the sensitivity of this technique is low, ranging from 30 to 70% for culture of skin biopsy specimens (11) to less than 5% for culture of cerebrospinal fluid (CSF) (81).

More than 100 research studies involving PCR in the diagnosis of *Borrelia burgdorferi* infections, with a variety of different target genes, primer pairs, PCR techniques, extraction procedures, and detection methods, have been published; however, only a few reviews have appeared on this topic (50, 80, 102, 133, 163).

In the first PCR paper, submitted for publication in April 1989 (146), a chromosomal gene, clone 2H1, which has recently been shown to code for a surface-associated 66-kDa protein (141), was used as target. Although primers were able to amplify 26 *B. burgdorferi* strains isolated in America, even in this first publication, it was shown that a German isolate (G2) failed to be amplified. Therefore, the primers had to be redesigned, and only in the second publication (145) was successful amplification of the German strain described. In 1990, Persing et al. described the amplification of *B. burgdorferi*-specific sequences from museum specimens (136) by using a target located on a 49-kb plasmid, coding for the outer surface proteins OspA and OspB. In the same year, Nielsen et al. (122) amplified a 145-bp DNA fragment of the OspA gene, Goodman et al. (52) were able to detect *B. burgdorferi*-specific sequences (*Ly* gene) in the urine of infected patients with late Lyme borreliosis, and Wallich et al. (181) sequenced the gene coding for flagellin, which later proved to be an excellent target for PCR (139). Lastly, in 1992, ribosomal 16S and 23S rRNA genes were selected successfully as targets (161).

METHODOLOGICAL ASPECTS

PCR is commonly used to amplify a unique region from a highly complex template such as genomic DNA. However,

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PCR is only one part of the so-called PCR assay. Other important aspects include DNA extraction and preparation and the amplification and detection of amplicons. Furthermore, controls which check for inhibition, contamination, and test standardization are essential.

Books (72, 73, 112, 135, 184) and many papers concerning basic protocols have been published. Therefore, this discussion will be focused strictly on PCR for *B. burgdorferi* detection.

Sample Preparation

In contrast to other bacterial and viral diseases, the number of organisms in clinical specimens appears extremely low in Lyme borreliosis. Although there can be up to 4,500 spirochetes in infected ticks (23), the number of genomes in the urine or plasma of infected patients is generally less than 50 per ml and rarely exceeds 5,000 per ml (50, 51). In CSF, the number of organisms might even be lower (93).

Depending on the specimen taken, various procedures have been developed for optimizing the amount of *B. burgdorferi* DNA available for amplification. In most studies, a procedure which included disruption of cells, inactivation of RNases and/or DNases with chaotropic salts, degradation of proteins with proteinase K, DNA extraction with phenol-chloroform, and precipitation with alcohols was used (25, 75, 84, 185). For efficient precipitation of small amounts of DNA, the addition of a carrier is essential. Glycogen (84, 123) has been used successfully, and the sensitivity of PCR could be increased by the addition of 10^3 *Leptospira biflexa* organisms (7) to CSF before centrifugation. In CSF, the addition of hyaluronidase can further increase the sensitivity of the PCR (96). Although the DNA prepared by precipitation is of high purity, these methods are very time-consuming and labor intensive and are difficult to use routinely.

Not surprisingly, procedures involving simpler methods have been developed; these include simple boiling of the specimen (88), centrifugation and boiling (31), alkali lysis (111), adsorption to coated or uncoated silica in the presence of chaotropic salts (7, 21, 51, 68, 105), boiling and concentration by centrifugation and ultrafiltration (128), and boiling in the presence of a cation exchanger (Chelex 100; Bio-Rad, Richmond, Calif.) (93). Although the mechanism by which Chelex improves the DNA extraction, is not known, it is thought to aid in stabilizing the DNA double helix (182).

DNA extraction procedures have been compared in a dilution study of *B. burgdorferi* with sterile water and CSF (97) and a few clinical studies with urine (68, 93, 105), CSF (68, 128), and synovial fluid (SF) (123) as specimens for PCR.

A comparative evaluation of four different methods of DNA isolation, i.e., (i) boiling; (ii) centrifugation, proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation; (iii) centrifugation, proteinase K digestion with Tween 20, and boiling; and (iv) solid-phase extraction with guanidine thiocyanate (Iso Quick; Microprobe, Garden Grove, Calif.), resulted in sensitivities of 0, 200, 5, and 5 spirochetes per ml of phosphate-buffered saline, respectively. When human body fluids were spiked with organisms, those that yielded the greatest sensitivity (10 spirochetes per ml) were plasma, SF, and CSF, followed by serum samples (20 spirochetes per ml), urine (50 spirochetes per ml), and whole blood (100 spirochetes per ml) (117).

PCR sensitivity was higher when fresh frozen skin specimens were used than when formalin-fixed, paraffin-embedded tissues were as specimens (186). Methods for DNA preparation from skin biopsy specimens or histological specimens have been reviewed recently (55). A general overview of DNA extraction procedures for PCR analysis is given by Panaccio et al. (130) and Greenfield and White (54).

TABLE 1. Published primers specific for the *ospA*/*ospB* gene of *B. burgdorferi*

Author	Yr of study	Method[a]	Position[b]	Reference(s)
Nielsen	1990	st	306–462	122
Malloy	1990	st	360–669	108
Persing	1990	st	301–946	136
Debue	1991	st	273–714	31
Yssel	1991	st	151–972	194
Guy	1991	n	334–894/362–713	57
Malawista	1991	st	165–425	107
Luft	1992	st	1123–1258	101
Duray	1992	n	301–700/566–700	37
Keller	1992	n	649–895	84
Hofmeister	1992	st	788–913	65
Williams	1992	n	124–893/222–856	189, 190
Liebling	1993	n	238–383/238–807	96
Rys	1993	st	149–349	149
Rys	1993	st	788–940	150
Persing	1994	st	1110–1411	134
Nocton	1994	st	149–343	123
Nocton	1994	st	788–943	123
Kawabata	1994	st	187–832	83
Moter	1995	n	168–829/204–595	116
Eiffert	1995	n	169–665/191–589	38

[a] PCR methods were either standard (st) or nested (n).
[b] Nucleotide positions given by the authors.

Target Selection

The most important consideration in selecting a target for amplification is genetic stability. Loss or alteration of the target sequence may result in a loss of reactivity. For the diagnosis of Lyme borreliosis by PCR, attention must also be paid to test specificity. Whereas genetically related pathogens such as *Borrelia hermsii* should not react in the test, all DNA subtypes of *B. burgdorferi* sensu lato that are pathogenic for humans, as well as novel uncharacterized isolates (8, 15), should be detected down to the theoretical limit of one organism.

A recent review has summarized the genome structure of *B. burgdorferi* (14). Most researchers in the United States have used the genes coding for the outer surface proteins, the *ospA* and *ospB* genes, which are located on a linear plasmid (Table 1). These genes are known to be highly variable (191) and have been found not to amplify all strains isolated in Europe (31, 84, 122, 137, 156). Although more than 20 different gene segments were amplified, only a few direct comparisons have been published. Whereas Nocton et al. (123) found no statistically significant difference between two *ospA* primer pairs, an increased sensitivity was found by Persing et al. (134) with *ospB* primers compared to *ospA* primers in tests of SF from infected patients. The sensitivity of PCR tests can vary by a factor of up to 10^3 for European strains of *B. burgdorferi* (116) with different *ospA* primers.

Sequences selected for amplification of the chromosomal flagellin gene are summarized in Table 2. Studies by Wallich et al. (181) and Picken (139) have shown that the amino-terminal and carboxy-terminal regions are highly conserved not only within *Borrelia* species and other spirochetes but also with *Bacillus subtilis* (181). Therefore, only the inner part of the flagellin gene is specific for *B. burgdorferi*. As no comparisons of different flagellin primers have been published, only a reference to our own work can be given. An increased sensitivity of PCR in clinical samples has always been achieved with a

TABLE 2. Published primers specific for the flagellin gene of *B. burgdorferi*

Author	Yr of publication	Method[a]	Position[b]	Reference
Persing	1990	st	121–320	137
Wallich	1990	st	128–852	181
Lebech	1991	st	52–842	94
Krueger	1991	st	271–560	88
Wiese	1991	st	50–468	193
Lebech	1992	st	594–842	93
Picken	1992	st	492–767	139
Luft	1992	st	442–630	101
Johnson	1992	n	245–858/300–689	76
Kawabata	1994	st	50–467	83
Persing	1994	st	107–342	134
Schmidt	1995	n	483–881/492–767	155
Wienecke	1995	n	547–740/562–726	186
Schmidt	1996	n	477–913/491–767	158
Trevisan	1996	st	592–666	176
Jaulhac	1996	st	475–707	74

[a] PCR methods were either standard (st) or nested (n).
[b] Nucleotide positions are those used by Gassmann et al. (46).

nested PCR assay. By carefully following guidelines (see the section on primer selection below), the annealing temperature could be increased from 52°C (155) to 55°C (158), resulting in a better performance.

A chromosomal gene (clone 2H1) has been found to be conserved in American strains (146). However, its inability to amplify a German isolate is well documented, and this could be overcome only by choosing different primers (145). Recently, it has been shown that one strain isolated in the Czech Republic also failed to amplify with primers specific for the clone 2H1 gene (128). By using clone 2H1 primers and a nested PCR system, 12 (80%) of 15 biopsy samples from nine patients with erythema migrans (EM) or acrodermatitis chronica atrophicans (ACA) were found to be reactive (114); a sensitivity of 69% was found in skin biopsy samples from 26 patients with EM (179). Serum specimens ($n = 12$), urine specimens ($n = 10$), and CSF specimens ($n = 2$) collected from 18 patients with neuroborreliosis were all reactive by PCR with nested primers specific for this chromosomal gene (32). Rabb et al. (142) demonstrated *B. burgdorferi* DNA in lesions of an antibody-negative patient with *Borrelia* lymphocytoma. Wallach et al. (180) were unable to amplify spirochetal DNA in the serum of seven patients with acute Lyme borreliosis. With the same gene as a target, but using a nested PCR generating an inner 92-bp amplicon (185), specific DNA could be amplified in formalin-fixed, paraffin-embedded skin lesions of 8 of 12 patients with EM.

Goodman et al. have sequenced the chromosomal *Ly1* gene (52), a fragment of the RNA polymerase C gene (51). They found that four of nine patients with late disease were excreting *B. burgdorferi* DNA in urine; SF from six of seven patients with Lyme arthritis (LA) were reactive with the same primers (22).

An increased sensitivity of PCR could be demonstrated in experiments in which *B. burgdorferi* was diluted into sterile water and CSF from normal donors. The primers of Goodman et al. (51) were used as inner primers in a nested PCR assay. By using specially designed outer primers, up to 10^3-fold-lower concentrations of *B. burgdorferi* DNA could be detected, compared with those detected by standard PCR methods (97). Using this nested PCR on urine specimens, 27 of 36 untreated patients with EM (75%) were found to have reactive specimens (156).

Based on GenBank studies, Schwarz et al. (161) examined a region near the 5′ end of the 23S rRNA sequence as a target for amplification. The unique tandem duplication of the 23S rRNA gene, observed in all *B. burgdorferi* isolates tested but not in other *Borrelia* species, was chosen as the second primer pair. Twenty-two skin biopsy specimens from patients with EM (59%) were positive by PCR compared with 57% by culture. 16S rRNA-specific primers have been used for rapid ribosequencing (6, 48) and gene detection (110, 121). However, in SF, rRNA primers were less sensitive than OspA primers (123, 134).

With a multiple-sequence alignment of 39 strains, representing all the genomic groups described in *B. burgdorferi* sensu lato, a highly sensitive nested PCR method was developed with primers homologous to conserved spots of the coding region of the *hbb* gene, encoding a conserved histone-like protein. This gene has not yet been tested on clinical specimens (177).

Primer Selection

The choice of oligonucleotide primers is a critical element in determining the sensitivity of PCR (63, 148). An optimal primer pair for detection of *B. burgdorferi* DNA should have the following characteristics. The pair should (i) amplify all strains of *B. burgdorferi* sensu lato; (ii) amplify all strains with the same sensitivity; (iii) be highly specific, not amplifying other borreliae, spirochetes, or other bacterial and viral pathogens; (iv) form stable duplexes with the template (all strains); (v) be 15 to 30 nucleotides long; (vi) have a high G+C content (the generally accepted value of 50% can hardly be obtained with *Borrelia* species, as the overall G+C content is 28% (69); (vii) not form 3′-end duplexes with themselves; (viii) not contain homooligomers or short repeated sequences; (ix) be stable at the 5′ termini but somewhat unstable at the 3′ ends; (x) be free of significant complementarity at their 3′ ends, as this promotes primer dimers; (xi) have melting temperatures that are very close to each other; and (xii) have a difference in melting temperatures with respect to the target that does not exceed 20°C, otherwise target self-annealing will predominate.

In practice, however, it is not possible to meet all the above conditions. The test specificity increases with elevated annealing temperature, e.g., longer primers, and primers with a higher G+C content. Computer programs for selecting optimal primers are available (34); however, they cannot replace practical experiments, especially in specificity studies.

Direct comparison of PCR results with different primers are given in a few studies with SF (22, 78, 107, 123, 134), CSF (32, 101), urine (32, 156), and skin biopsy (35, 186) specimens. PCR results with chromosomal primers in SF were less sensitive than were results with plasmid primers, despite a similar analytical sensitivity (123, 134). No statistically significant differences between primer pairs were seen in CSF and skin specimens. However, with primers generating long amplicons (730 bp) compared to short ones (164 bp), reactivity in formalin-fixed, paraffin-embedded tissue samples was reduced, probably because of extensive degradation of DNA during the process of fixation in formalin (186).

Selection of the PCR Method

Theoretically, PCR can generate 10^9 amplicons after 30 cycles. This is more than sufficient for detection even by simple visualization in agarose gels. However, in practice, an efficiency of 1.0 is not achievable, and further increasing the cycle number does not increase amplicon yield because of the plateau effect (71).

Sensitivity in clinical samples can be lowered by losses dur-

ing DNA preparation, by the presence of extraneous DNA (27, 111), or by contamination with interfering and/or inhibitory substances (see the section on control of inhibition, below). One way to increase the sensitivity of PCR is by using a nested PCR procedure. Here, two rounds of amplifications are performed. After a first standard PCR, a small sample (1 to 3 μl) is removed and put into a master mix containing primers specific for an inner part of the generated amplicon. After a second round of amplification cycles, the sensitivity is increased, as expected (58) (this procedure was recently shown to be up to 10^3 times [87] or 10^5 times [177] more sensitive). In addition, by using two sets of specific primers rather than one, the specificity is improved, possibly making a hybridization detection step unnecessary (192). As a consequence of the improved sensitivity of nested PCR methods, less sensitive detection methods, e.g., ethidium bromide-stained agarose gels, can often be used.

Although nested PCR is superior in both sensitivity and specificity to a standard PCR, the technique is much more prone to contamination. Contamination problems can be overcome by strictly following published precautions for avoiding false-positive results (90, 155). However, one must consider that all published studies with nested PCR have been performed in specialized, mostly research laboratories, and up to now companies selling PCR kits for the detection of pathogens have avoided using this technique in their assays.

Another solution for avoiding contamination with nested PCR is the single-tube nested PCR technique (39, 127, 131, 174). The most critical step in performing a nested PCR, i.e., opening the reaction tube after the first PCR, is not necessary here. Instead of amplifying the DNA in two separate tubes, both the first and second PCRs with outer and inner primers are done in one reaction vessel. The two PCR products are separated thermodynamically by designing outer primers with a high annealing temperature and inner primers with a low annealing temperature. Cycling in the first PCR is done within the range including the annealing temperature of the outer primers and the denaturation temperature of the amplicon. Within this temperature range, the inner primers have no chance to anneal in the first round of amplifications. In the second round, the temperature is decreased to the annealing temperature of the inner primers. In addition, it is best to also lower the concentration of the outer primers to at least 1/50 that of the inner primers. This technique has been shown to simplify the amplification and diagnosis of viral (174) and bacterial (131) infections. One report has been published recently on *B. burgdorferi* (138).

Detection

Traditionally, PCR-generated products are analyzed by gel-based methods. Agarose gel electrophoresis with ethidium bromide staining is simple and inexpensive but suffers from a lack of sensitivity and specificity. Starting with 10 initial target molecules in a 100-μl amplification reaction, a 200-bp PCR product will become visible only after 32 cycles of 100% efficiency (91), a level of amplification that is often not achievable with clinical samples. To overcome this problem, PCR products separated in an agarose gel are transferred to a membrane by standard blotting techniques and are subsequently detected with a labelled probe with a specific sequence. Most studies cited have used autoradiography with ^{32}P-labelled probes. Other detection methods used in PCR for *B. burgdorferi* include digoxigenin probes (7, 22, 128) and chemiluminescent probes (134). In addition, modified PCR products generated by biotin-labelled primers (100) or polybiotinylated detection probes (109) have been used with a capture assay.

Sensitivity

In many of the published studies, the sensitivity of a given protocol was tested by analysis of appropriately prepared *B. burgdorferi*-spiked samples. However, the detection limit of less than 10 organisms does not necessarily predict a similar level of clinical sensitivity (93, 123). The test sensitivity is dependent on technical details and the particular specimen used for analysis. Some patients with arthritis can harbor up to 10,000 spirochetes per ml of SF (22), and patients with EM and systemic symptoms can harbor more than 4,000 organisms per ml of plasma (51). However, one should be aware that the number of spirochetes in human body fluids is generally low (<50 organisms per ml), especially in late, chronic disease.

Besides the selection of a proper PCR method, test sensitivity is influenced mainly by the preparation of DNA, presence of extraneous DNA, inhibitory substances, and detection procedures. Interference by host DNA with the detection of *B. burgdorferi* DNA by PCR was shown in a study with rhesus monkeys (27). The specific amplicon was not detectable if more than 500 ng of monkey skin nucleic acid was included in the PCR mixture. With less than 500 ng of extraneous DNA, the detection limit of PCR was 10 spirochetes.

Generally, for determining sensitivity, one requires a "gold standard," which for most pathogens is culture. However, in Lyme borreliosis, culture often fails as a gold standard. In addition, given the difficulty in making a clinical diagnosis, especially in late disease, this is a poor choice of comparative standard as well. One exception is EM, a typical skin lesion seen in the early stage of the disease. This clinical sign can easily be recognized, and a high sensitivity of PCR has been documented in untreated patients with EM (32, 89, 155, 161).

Although *B. burgdorferi* DNA could be detected in human body fluids from patients with late disease even 10 years or more after infection (22, 75, 84, 93, 96, 114, 123, 155), the inability of PCR to confirm late disease has also been reported (38, 68, 107, 128, 195). Therefore, another way to define sensitivity might be to establish an expanded gold standard. Here, culture-negative but PCR-positive patients are tested by a second PCR targeting a different gene. However, only a few studies have compared PCR with different genes (32, 35, 78, 101, 107, 123, 156, 186), and the results are not always in accord. Sampling errors and DNA preparation methods do affect an individual assay when amplifying specimens with low numbers of target molecules, i.e., <20. Therefore, PCR should be performed on multiple aliquots.

Specificity

The specificity of PCR is imparted mainly by (i) specific primers and (ii) the specific probe. In addition, the concentration of reagents of the PCR mix (deoxynucleoside triphosphates, *Taq* polymerase, primers, magnesium chloride), as well as the selected PCR method (e.g., nested PCR [97] or the hot-start technique [18]), can influence specificity. The cross-reactivity of selected oligonucleotides can be checked by searching databases for sequences that are available; however, closely related bacteria (52, 139, 146) must always be tested experimentally. Furthermore, a PCR that uses urine as DNA source must be checked for reactions with common urinary tract pathogens (155, 156). If CSF is used as the specimen, viral and bacterial pathogens known to be involved in central nervous system (CNS) infections (7, 88, 101) should be tested for cross-reactivity.

Control of Inhibition

Inhibitors of PCR are frequently detected in clinical specimens. Extraction and precipitation of DNA with phenol-chloroform-ethanol reduces inhibition (65, 84), although the opposite results have also been reported (93, 128). PCR was inhibited for 7 of 10 urine samples and 4 of 10 CSF samples when DNA was prepared by protein K treatment, phenol-chloroform extraction, and ethanol precipitation. However, when DNA was denaturated with sodium iodide and adsorbed on glass beads (Geneclean kit; B01-100, La Jolla, Calif.) (68), only 1 of 10 urine samples and 0 of 10 CSF samples from the same patients were inhibited. In the preparation of DNA from urine with Chelex-100, inhibition was reduced to 2% of 326 samples tested (155).

To be certain that negative results are not due to inhibitory factors in clinical fluids, so-called housekeeping genes are generally coamplified. Each negative sample is checked with primers specific for human genes. If amplification fails, the enzyme is considered to be inhibited and these specimens cannot be analyzed by PCR. The genes of human leukocyte common antigen, CD45RA (96), the APOE C1-C2 gene cluster on human chromosome 19 (31), the β-globulin gene (41), the glyceraldehyde phosphate dehydrogenase gene (35), and the gastrin gene (93) have been used as controls in *B. burgdorferi* PCR.

One possible limitation of using housekeeping genes as inhibition controls involves the different conditions needed to amplify *B. burgdorferi* and these control genes. The reaction conditions of PCR are often appropriate for only one gene. To avoid these difficulties, competitive DNA fragments, in which only the nucleotides at both ends are complementary to *B. burgdorferi* gene, can be constructed (162). These internal standards have the same kinetics of reaction and react with the same *B. burgdorferi*-specific primers, but they differ only in their nucleotide length to be easily differentiable from the specific amplicons (155). Sometimes, the addition of a known amount of *B. burgdorferi* (123), as well as a simple dilution of prepared DNA (93), can be used to detect inhibition.

Control of Contamination

One of the major problems of PCR-based assays, namely, the extremely high sensitivity, is also its greatest advantage. Any contaminant, even the most minute airborne remnant carried over from the previous PCR procedure, may be multiplied and can give false-positive results. Precautions for avoiding amplicon carryover (reviewed in reference 90) must be strictly followed. In addition, two amplicon sterilization methods have been evaluated: (i) a post-PCR method with a psoralen derivative and long UV light exposure (this method is efficient only with amplicons greater than 300 bp [40, 132]) and (ii) an enzymatic method in which dUTP is substituted for dTTP and amplicons are cleaved with uracil-*N*-glycosylase (150, 195). The latter method has been shown also to enhance the enzyme-linked immunosorbent assay signal in quantitative detection assays (100). However, neither method can be used with nested PCRs. One approach to overcome contamination problems with nested PCR is the use of the new single-tube nested PCR technique (see the section on selection of PCR methods, above).

Advantages and Limitations of PCR Assays

The advantages of PCR for the diagnosis of *B. burgdorferi* infections are its speed, its high degree of sensitivity and specificity, and its cost-effectiveness. The ability to detect one copy of a specific sequence of *B. burgdorferi* DNA has made PCR methods attractive in the laboratory diagnosis of Lyme borreliosis. Tissues and body fluids can be used as the starting material for PCR; however, details of patient sample requirements and rapid sample preparation techniques remain to be worked out.

There are two major methodological hurdles in the optimal use of PCR technology for the diagnosis of Lyme borreliosis. The greater problem is that of false positivity due to contaminating nucleic acids, especially the accumulation of PCR products (amplicons) in the laboratory by repeated amplification of the same target sequence. Amplicon contamination is extremely troublesome in assays tuned for maximum sensitivity, a necessity for the diagnosis of *B. burgdorferi* infections. To avoid amplicon carryover, laboratories performing PCR must take specific precautions (see the section on control of contamination, above). These precautions can be adopted by research laboratories, but they represent a severe limitation to service diagnostic laboratories.

The second methodological barrier is the changeable nature of borrelial DNA, which results in potential false-negative tests because of sequence differences in the target gene. Outer surface protein sequences in *B. burgdorferi* are extremely variable, and significant genetic differences tend to be present between European and American strains rather than among American isolates (see the section on target selection, above). However, as more and more specific sequences become available in databases, it will become easier to select optimal, conserved DNA sequences for amplifications.

DIAGNOSTIC ASPECTS

PCR results are dependent on the concentration of spirochetes in the specimens taken for analysis. The sensitivity of PCR of skin biopsy specimens from patients with EM is high (161), but the procedure is invasive and cannot be used routinely. In blood, spirochetemia is transient, like the bacteremia in most other infections, and high detection rates can be expected only during a short period of primary infection. Due to the ability of *B. burgdorferi* to bind to activated platelets within the host (26), the number of organisms is higher in plasma than in serum, perhaps because they are trapped by the clotting process (51).

Excretion of *B. burgdorferi* or *B. burgdorferi* DNA into urine was demonstrated by Goodman et al. (52) in 1991 in eight patients with late disease; studies with higher patient numbers have been reported only recently. Urine can be a suitable specimen for diagnosis; however, little is known about how spirochetes invade the urine or if whole borreliae are present in human urine, over though borreliae have been previously demonstrated in the urine of infected mice. By using a method in which soluble DNA is adsorbed to glass beads without previous enzymatic digestion or boiling, it was recently shown that soluble DNA or antigen-associated DNA is excreted into the urine (105).

In the following sections, PCR results in tests of patient specimens including skin biopsy specimens, blood or serum, and urine are discussed. In addition, SF and CSF from patients with neuroborreliosis and joint manifestations have been tested. These results are summarized in Tables 3 to 7.

B. burgdorferi DNA was also amplified from the ligament tissue of a patient with chronic Lyme borreliosis (61), a benign skin infiltrate (142), and the breast milk of lactating women with EM (155).

TABLE 3. Results with skin biopsy specimens or cultures of skin biopsy specimens for *B. burgdorferi* PCR

Gene	Method[a]	Ann. temp[b] (°C)	No. of cycles (st/n)[c]	Amplicon size (bp)[d]	No. of patients reactive/no. tested	Comments[e]	No. of control patients reactive/no. tested	Reference
Chromosomal								
Flagellin	n	48	40/30	290	20/20	Patients with morphea (9), LSA (6), EM (4), and ACA (1)	0/4	151
	n	50	30/25	290	1/1	Patient with osteomyelitis		126
	n	37	40/40	192	0/30	Patients with morphea (20), and LSA (10)		35
Clone 2H1	n	37/35	30/30	126	7/9	Patients (reactive/tested) with EM (3/4), and ACA (4/5)		114
	n	50	30/30	92	9/13	Patients (reactive/tested) with EM (8/12), and LB (1/1)	0/4	185
	n	50	30/30	92	29/32	Patients (reactive/tested) with EM (24/27), and ACA (5/5) in fresh frozen tissue		186
	n	37/35	30/30	126	40/62	Patients (reactive/tested) with EM (18/26), and ACA (22/36)	0/4	179
rRNA	st	50	25	576	7/7	Patients with EM, biopsy taken 1–6 mo after disappearance of EM lesion from healthy-appearing skin		89
	st	43	35	260	21/37	Cultures of skin biopsy specimen taken from patients with EM		161
	st	43	35	260	20/36	Cultures of skin biopsy specimen taken from patients with EM	0/13	160
	st	48	35	260	0/31	Patients with morphea		41
Plasmid								
ospA/B	n	45/55	30/25	392	19/22	Patients with EM (8/10), and ACA (11/12) (sensitivity in strains can differ up to 10^3)	0/4	116
	st	60	25	98	0/14	Patients (reactive/tested) with EM (6/7), and morphea (0/7)	0/8	143

[a] n, nested; st, standard.
[b] Annealing temperature.
[c] Cycles used in the standard (st) or nested (n) PCR method.
[d] Size of the generated amplicon.
[e] LSA, lichen sclerosus et atrophicus; LB, Lyme borreliosis. The number of patients is given in parentheses.

Patients with Dermatological Manifestations

EM, a typical and distinct lesion that appears in the initial phase of infection, can be observed in up to 60% of patients in Europe (166) and 60 to 80% of patients in the United States (167). Not surprisingly, skin biopsy specimens of the lesion have been the first samples to be examined by PCR. The data are summarized in Table 3. Skin biopsy specimens were taken from the advancing border of the EM lesion. *B. burgdorferi*-specific DNA could be detected in 8 of 12 formalin-fixed, paraffin-embedded skin specimens from patients with EM (185). A test sensitivity of 60 to 80% was obtained by using nested PCR with frozen skin tissues (Table 3). Interestingly, positive results were sometimes found only when the DNA preparation was diluted 1:10 or 1:100, indicating an inhibitory effect (179). In a Finnish study, six of seven patients with untreated EM (culture positive) were PCR reactive in a standard PCR with primers specific for the *ospA* gene (143). However, nonspecific amplification products were common, and these strains isolated in Finland failed to hybridize with a probe specific for an American strain (143). The detection rate of *B. burgdorferi* DNA by PCR was higher in frozen skin tissues (90%) than in paraffin-embedded skin specimens (44%) from patients with EM (186).

When skin biopsy specimens are first cultured and the culture material is analyzed by PCR, the test sensitivity should be higher because of the multiplication of organisms in culture. In one such study in which a nested PCR targeting the *ospA* gene was used, 8 of 10 patients with EM were positive; the interval between the tick bite and the presentation of the patients varied between 1 week and 3 months. The sensitivity of PCR (80%) was considerably higher than that of culture (14%) or serologic testing (77%) (116).

With primers specific for the 16S and 23S rRNA genes, the overall sensitivity of PCR of cultures from skin biopsy specimens was 59%, similar to that of culture (57%). In addition, five of eight cultures that were overgrown by contaminating organisms and could not be evaluated were PCR positive (160). After 2 weeks of culture, 14 of 20 skin biopsy specimens were positive by microscopic examination; with PCR, 19 of 20 were reactive. Two patients became positive by culture after more than 10 weeks; in contrast, PCR positivity could be demonstrated beginning from the second day of culture (161). In a second study, all four patients with EM were PCR positive when skin biopsy specimens from the edge of the area of EM were analyzed; samples from the center were negative. PCR of the vesicle fluid (vesicular EM) gave positive results (49).

The demonstration of specific DNA in the blood of patients with EM might be hampered by the presence of inhibitors, as only two of five patients were positive by PCR (57). In a second study performed with supernatants of cultures from blood and serum, PCR was negative in all seven patients examined (180).

Recently, plasma from 76 patients with EM was analyzed in a prospective, blinded study of PCR (51). Spirochetemia could

TABLE 4. Results with blood or serum as specimens for *B. burgdorferi* PCR

Gene	Sample vol (ml)	Method[a]	Ann. temp[b] (°C)	No. of cycles (st/n)[c]	Amplicon size (bp)[d]	No. of patients reactive/no. tested	Comments[e]	No. of control patients reactive/no. tested	Reference
Chromosomal									
Clone 2H1	3	st	68	35	127	10/10	Patients with NB		32
Polymerase C	0.5	st	50	39	232	14/76	Patients with EM	0/29	51
Plasmid									
ospA/B	0.1	n	37	30/35	332	2/5	Patients with EM		57
	1	n	55	35/25	146	13/25	Patients (reactive/tested) with EM (3/5), carditis (2/2), peripheral neuropathy (0/1), CNS disease (3/6), and LA (5/11)	0/3	96
		n	47	40/30	93	9/22	Patients reactive with EM (3), Ball's palsy (3), arthralgias (3)	0/10	117
	0.5	n	52	30/40	641	2/2	Patients with LA		189
	3	st	61	35	308	10/10	Patients with NB		32

[a] n, nested; st, standard.
[b] Annealing temperature.
[c] Cycles used in the standard (st) or nested (n) PCR method.
[d] Size of the generated amplicon.
[e] NB, neuroborreliosis. The number of patients is given in parentheses.

be documented in 14 patients (18.4%) by PCR (Table 4); only 4 (5.3%) were culture positive. Positive PCR results correlated with clinical evidence of disseminated disease: 10 of 33 patients with systemic symptoms (30.3%) were positive compared with 4 of 43 without such evidence (9.3%).

A new method for confirming the clinical diagnosis of EM was presented recently (155). Analysis of urine samples by a nested PCR targeting a specific part of the flagellin gene showed that 88 of 97 patients with EM were positive (Table 5). Within the first week the lesion was present, 2 of 4 patients tested were reactive, but the sensitivity decreased when the lesion was more than 7 weeks old (155). These results were confirmed in a second study with infected patients from a different geographic region (158).

Breast milk and urine from two women with EM, one with the lesion on the shoulder and the other with the lesion on the breast, who had delivered and were nursing their babies, were tested by PCR, and both specimens were highly positive (155).

TABLE 5. Results with urine as the specimen for *B. burgdorferi* PCR

Gene	Sample vol (ml)	Method[a]	Ann. temp[b] (°C)	No. of cycles (st/n)[c]	Amplicon size (bp)[d]	No. of patients reactive/no. tested	Comments[e]	No. of control patients reactive/no. tested	Reference
Chromosomal									
Flagellin	0.2–0.5	n	50	30/25	290	1/10	Children with NB	0/25	68
	0.2	n	50	30/25	290	13/59	Patients (reactive/tested) with LA (9/24), and NB (4/35)		79
	5	st	39	40	248	5/10	Patients with NB, PCR in urine more sensitive than in CSF!		93
	3	n	52/52	25/35	276	88/97	Patients with EM	2/49	155
	3	n	55/55	25/35	277	22/24	Patients with EM	3/62	158
Clone 2H1	3	st	37	35	127	10/10	Patients with NB		32
Polymerase C	0.5	st	50	39	225	4/8	Patients with LD		52
rRNA	0.35	st	50	45	259	17/26	Patients with EM (1), cardiac (3), neurological syndromes (7), and joint manifestations (15)		105
Plasmid									
ospA/B	1	n	55	35/25	146	3/3	Patients with NB	1/13	96
		n	52	30/40	649	2/2	Patients with LA		189
	3	st	68	35	190–544*[f]	10/10	Patients with NB		32

[a] n, nested; st, standard.
[b] Annealing temperature.
[c] Cycles used in the standard (st) or nested (n) PCR method.
[d] Size of the generated amplicon.
[e] NB, neuroborreliosis; LD, late disease. The number of patients is given in parentheses.
[f] Depending on the strain.

In one of the women, *B. burgdorferi* was cultivated from a skin biopsy specimen.

Borrelia lymphocytoma, another early manifestation of Lyme borreliosis, presents preferentially on the earlobes of children or the nipple region of infected adults. Specimens taken from infected skin of such patients were tested by PCR (143, 185). Using urine as the specimen, all five patients tested were found positive (155). One child with a lymphocytoma on the right earlobe was retested by PCR of a urine sample 5 days after the beginning of treatment, and the test was nonreactive. In contrast, excretion of *B. burgdorferi* DNA into the urine of 32 of 56 patients with EM was still detected by PCR immediately after the end of approximately 2 weeks of therapy (155).

ACA is a late dermatologic manifestation of Lyme borreliosis; nevertheless, the PCR sensitivity (61 to 91%) in skin specimens is comparable to that in EM patients (114, 116, 119, 151, 153, 179). Surprisingly, in four of five patients with histologically proven disease, *B. burgdorferi* DNA could also be found in urine specimens (155). In one patient, PCR of a frozen skin section of the lesion was positive, even though the onset of the disease had been 10 years earlier (114). In all specimens of patients with ACA, only *B. afzelii* was detected. These data indicate that ACA might be closely associated with this genospecies (12, 125, 187).

Some types of morphea or circumscribed scleroderma are thought to be caused by *Borrelia* infection (2). In Finnish (143) and German (186) studies, specific DNA could not be amplified in any of 6 or 30 patients, respectively. However, samples of skin lesions from patients with morphea were positive by PCR in other German studies (151, 152, 183) and in 6 of 10 patients in Italy (176). In North America, *B. burgdorferi* DNA was undetectable in patients with morphea, as shown by tests with different sets of primers (33, 35, 41, 113). These negative findings might be because *B. burgdorferi* sensu stricto, the predominant strain in North America, has never been found associated with late dermatological manifestations of Lyme disease.

Recently, several reports have indicated that besides circumscribed scleroderma, two other skin diseases might be caused by infection with *B. burgdorferi*. In granuloma anulare, *B. burgdorferi* was demonstrated on histological sections with a polyclonal antiserum (3) and in culture from a skin biopsy sample (173). Wienecke et al. (185) could not amplify specific DNA from formalin-fixed, paraffin-embedded skin biopsy specimens. However, in another study (4), the excretion of *B. burgdorferi* DNA in urine could be demonstrated. With two independent nested PCRs specific for the chromosomal flagellin and the *Ly* gene, urine samples from 8 of 13 patients with granuloma anulare gave positive results. A previous tick or insect bite or arthralgias were reported only for PCR-positive patients.

The pathogenesis of lichen sclerosus et atrophicus is unknown, and there are only a few reports that consider the etiology to be *B. burgdorferi*. Borreliae were detected immunohistochemically in skin sections of lesions (5, 147). Specific DNA could be amplified from 6 of 6 and from 3 of 3 skin biopsy specimens (151, 175) and from 13 of 19 urine specimens (4) from infected patients. To prove the specificity of PCR with flagellin as the target, the chromosomal *Ly* gene was also tested and similarly found to be positive. These findings provide evidence that *B. burgdorferi* may be a pathogenic factor in a subgroup of patients with granuloma anulare and lichen sclerosus et atrophicus, at least in a limited number of patients studied in Europe.

Furthermore, PCR techniques have been used to demonstrate *B. burgdorferi* DNA in tissues of patients with dermatomyositis (45, 64, 67) and in patients with eosinophilic fasciitis (Shulman's syndrome) (53, 60).

Patients with Neurological Manifestations

The broad range of sensitivity found in PCR studies of CSF samples (Table 6) can be explained by (i) the selection of patients and (ii) the extremely low levels of organisms present in CSF. In most studies, the detection limit was between 20 and 50 spirochetes per ml of CSF (25, 68, 75, 93). Nevertheless, the results in Table 6 indicate that this level can be too high for detection of infection in vivo. Borrelial DNA concentrations in CSF may vary over several orders of magnitude among patients with neuroborreliosis (84); however, no quantitative data are yet available. Low numbers of spirochetes (<20 organisms per ml), resulting in borderline PCR results, might also be responsible for the lack of reproducibility. In one study, the CSF sample from one patient was tested 13 times, giving 7 positive and 6 negative PCR results (84).

Concerning the selection of patients, it is evident that patients with meningitis, polyneuritis, meningoencephalitis, and meningoradiculitis can be more readily found positive by PCR than patients in the later stages of disease. These findings are in agreement with culture results. *B. burgdorferi* has been cultured from the CSF of a few patients with second-stage (early) neuroborreliosis (81, 169) but never from patients with third-stage (late) neuroborreliosis (93).

PCR detected *B. burgdorferi ospA* DNA in the CSF of 10 of 11 patients with Lyme encephalopathy, 28 of 37 patients with CNS disease (lymphocytic meningitis [1 patient], facial nerve palsy [4 patients], radiculitis [3 patients], multifocal encephalomyelitis [13 patients], spastic paresis [9 patients], relapsing or remittening multiple sclerosis-like disorders [6 patients], cervical myelopathy [1 patient]), and 0 of 23 controls (84).

A French group found that 11 of 45 patients with meningoradiculitis and/or facial palsy (24%) were positive by PCR of CSF; all the patients had CSF pleocytosis and positive serologic test results and showed improvement after antibiotic therapy (7). In a second, prospective study with 225 CSF samples from patients in whom the diagnosis Lyme borreliosis was considered by a clinician, the detection rate was increased from 3 to 14% by using an improved DNA preparation (7).

In other studies, the high sensitivity found in all 10 patients tested (8 with meningoradiculitis and 2 with meningoencephalomyelitis) (31), in 5 of 6 patients (2 with meningitis, 2 with polyneuritis, and 1 with meningoencephalitis) (75), in 4 of 6 patients (cranial neuritis) (101), and in 13 of 14 patients (with CNS manifestation but no further data) (96) might be attributable to the careful selection of the patients.

Luft et al. (101), using three independent PCRs with hybridizations for flagellin, *ospB*, and 16S rRNA sequences, found that for 8 of 12 patients with infections for less than 2 weeks, the CSF PCR result was positive. In addition to the references listed in Table 6, other authors have reported performing PCR of CSF in patients with neuroborreliosis (13, 20).

Children with neuroborreliosis generally have a much lower PCR reactivity in CSF, and diagnosis is therefore as difficult as with serologic testing (43). *B. burgdorferi* DNA could be detected in two of seven children with facial palsy (25), in two of eight with peripheral seventh-nerve palsies without intrathecal antibody synthesis (68), and in one of eight with facial palsy, all with intrathecal antibody synthesis (195). In addition, *B. burgdorferi* DNA was detected in one child with Guillain-Barré syndrome (66).

The negative or intermediate PCR results obtained for a significant percentage (54%) of patients with definite or prob-

TABLE 6. Results with CSF as the specimen for *B. burgdorferi* PCR

Gene	Sample vol (ml)	Method[a]	Ann. temp[b] (°C)	No. of cycles (st/n)[c]	Amplicon size (bp)[d]	No. of patients reactive/no. tested	Comments[e]	No. of control patients reactive/no. tested	Reference
Chromosomal									
Flagellin	0.2	st	39	40	248	4/10	Patients with NB	0/25	93
	3	st	48	35	290	2/2	Patient with meningitis and cranial nerve palsies, 1 child with facial palsy	0/2	88
	0.2–1.5	n	50/51	30/25	290	2/8	Children with NB, 4/8 were antibody negative		68
	0.5	Ref 68				2/12	8 children, 4 adults with NB	0/15	195
	0.5–1	st	55	40	230	5/6	Patients with meningitis (3), polyneuritis (2), meningoencephalitis (1)	0/10	75
		ND				1/1	Child with NB		66
	0.05	st	55	35	188	8/12	Patients with acute, disseminated LB	0/16	101
	3	n	52/52	25/35	276	2/2	Patients with NB		155
Clone 2H1	0.4	n	37/37	30/30	294	11/24	Patients with definite or probable NB	1/33	128
	3	st	37	35	294	2/2	Patients with NB		32
rRNA	0.05	st	55	35	169	8/12	Patient with acute disseminated LB; (*B. hermsii* also reacts)	0/16	101
Plasmid									
ospA/B	1	n	55/55	35/25	146	13/14	Patients with neurologic manifestations	1/14	96
		st	60	39	176	10/10	Patients with meningoradiculitis (8) and meningoencephalomyelitis (2)	0/3	31
	0.2	n	39	35/25	186	45/55	Patients (reactive/tested) with Lyme encephalopathy (10/11), and inflammatory CNS disease (28/37)	0/23	84
	0.05	st	55	35	133	8/12	Patients with multiple EM (4/6), and cranial neuritis (4/6)	0/16	101
	0.2	n	59	40		2/7	Children with facial palsy	0/20	25
	3	st	65–69	35	345	1/2	Patients with NB (one patient reactive only with *B. garinii*-specific primers)		32
	0.2	n	59/61	15/25	399	12/63	Children with acute NB[d] (all IgM positive)		38
	1	st	50	30	333	11/45	Patients with NB	0/40	7

[a] n, nested; st, standard; ND, no data available.
[b] Annealing temperature.
[c] Cycles used in the standard (st) or nested (n) PCR.
[d] Size of the generated amplicon.
[e] NB, neuroborreliosis; LB, Lyme borreliosis. The number of patients is given in parentheses.

able Lyme borreliosis were attributed to the absence or extremely low levels of spirochetes (128).

Finally, the rather low sensitivity of PCR in CSF specimens from patients with late disease might be because spirochetes adhere to tissues. So far, no studies comparing the number of *B. burgdorferi* organisms in brain tissues, especially the meninges versus the CSF of infected animals, have been done. In comparison, PCR studies with CSF of patients with neurosyphilis, another spirochetal disease, have shown a reactivity of about 50% (56, 62, 124), although the spirochetal load is higher in neurosyphilis patients. In patients with early syphilis, *Treponema pallidum* DNA could be repeatedly found in preparations from buffy coat cells, but sera and, to a lesser extent, plasma from the same patients were nonreactive in PCR (157). These findings correspond well to studies in which Treponema pallidum was found adhering to cells or tissues (44).

An interesting phenomenon was documented by Lebech and Hansen (93). By comparing PCR results with CSF and urine of patients with neurosyphilis, they found a higher detection rate in urine (5 of 10 pretreatment samples) than in CSF (2 of 10), although eight patients had lymphocytic meningoradiculitis and two patients had chronic progressive encephalomyelitis. The diagnosis of neuroborreliosis was based on lymphocyte pleocytosis and specific intrathecal immunoglobulin M (IgM) and/or IgG antibody synthesis. As the analytical sensitivity of their PCR was equally high in both body fluids, they explained the results as being due to a lower number of *B. burgdorferi* genome copies in CSF. This assumption is supported by pre-

TABLE 7. Results with synovial fluid as the specimen for *B. burgdorferi* PCR

Gene	Sample vol (ml)	Method[a]	Ann. temp[b] (°C)	No. of cycles (st/n)[c]	Amplicon size (bp)[d]	No. of patients reactive/no. tested	Comments	No. of control patients reactive/no. tested	Reference
Chromosomal									
Flagellin		n	50	30/25	290	1/1	Child with LA		78
	0.5	st	62	40	230	4/11	Patients with LA	0/29	74
	0.1–0.2	st	50	50	256	9/17	Patients with LA (culture negative)	0/11	134
Polymerase C	0.5–1	st	50	39	231	6/7	Patients with LA	0/38	22
rRNA	0.2	n	50	50	368	42/88	Patients with LA	0/57	123
	0.1–0.2	st	50	50	368	0/20	Patients with juvenile rheumatoid arthritis		107
	0.1–0.2	st	50	50	368	8/18	Patients with LA (culture negative)	0/11	134
Plasmid									
ospA/B	0.2	n	39	35/25	186	1/1	Patient with LA		19
	0.5–1	st	50	55	156	6/7	Patients with LA	0/39	22
	0.5	n	50	30/25	290	1/1	Child with LA		78
	1	n	55	35/25	146	4/5	Patients with LA	0/20	96
	0.1–0.2	st	50	50	158	0/20	Patients with juvenile rheumat. arthritis		107
	0.2	st	50	45	3 sets	75/88	Patients with LA	0/64	123
	0.2–0.3	st	50	50	200/328	18/18	Patients with LA (culture negative)	0/11	134
		st	60	39	176	3/3	Patients with LA	0/3	31

[a] n, nested; st, standard.
[b] Annealing temperature.
[c] Cycles used in standard (st) or nested (n) PCR method.
[d] Size of the generated amplicon.

viously successful detection of *B. burgdorferi* antigens in urine from experimentally infected animals (36, 70, 103).

Patients with Joint Manifestations

Approximately 60% of untreated patients in North America infected with *B. burgdorferi* have brief, intermittent attacks of arthritis that may recur for several years (171). A small percentage of these patients have continuous arthritis for 1 year or longer, a condition called chronic LA (168).

LA can usually be treated successfully with either a 1-month course of doxycycline or a 2-week course of ceftriaxone (170). However, a small percentage of patients have persistent arthritis despite multiple courses of oral and intravenous antibiotic therapy. It has been unclear whether this treatment-resistant course results from persistent infection or from a postinfective immune system-mediated phenomenon. The demonstration of viable *B. burgdorferi* in joint fluids could answer that question; however, *B. burgdorferi* has been recovered by culture from the synovial fluid of only two patients with LA (154, 165).

PCR studies of synovial fluid of patients with LA are summarized in Table 7. Very often only a few patients were tested, and the sensitivity varied from 0 of 20 patients with juvenile rheumatoid arthritis (107) to 2 of 5 (96) and 6 of 7 (22) patients with LA.

Nocton et al. have tested stored SF samples from 127 patients with LA, collecting during a 17-year period (123). *B. burgdorferi* DNA was detected in SF from 75 of 88 LA patients and none of 64 control patients. PCR reactivity correlated significantly with higher leukocyte counts in SF, shorter durations of illness and arthritis, and a longer duration of arthritis after aspiration. Of 73 LA patients who were untreated or treated with only short courses of antibiotics, 70 (96%) had positive PCR results. The high sensitivity could be obtained only with OspA primer-probe sets; in contrast, the primer-probe set that detected chromosomal DNA was less sensitive. This discrepancy was further evaluated with 19 SF samples from 18 patients with LA, all of which were culture negative (134). DNA sequences characteristic of plasmid-encoded genes were detected in all 19 samples. However, only 8 of the 19 samples were reactive with primers specific for the rRNA gene and 9 of 17 samples were positive with primers specific for the flagellin target, despite the superior analytical sensitivity of the chromosomal PCRs. This apparent overrepresentation of *B. burgdorferi* plasmid sequences was found exclusively in clinical samples (SF samples) and not in cultured organisms and was referred to as target imbalance (134).

In a study of PCR of the chromosomal flagellin gene in synovial tissue and SF, 10 synovial tissue samples from 11 patients with LA were positive, whereas SF samples were positive in only 4 (74).

In addition to reports summarized in Table 7, *B. burgdorferi* DNA was detected by PCR in blood (189), SF (140, 165), CSF (101), and urine (52, 79, 105, 155, 189) of patients with LA. Maiwald et al. (105) found that 11 of 15 patients with LA (1 with arthralgia and a typical history of a tick bite and EM, 9 with arthritis as a major symptom with other etiologies ruled out, and 5 with arthritis in combination with other manifestations) were reactive on tests of urine by a PCR that amplified a 259-bp fragment of the 23S rRNA gene (161). A flagellin-specific DNA could be amplified in urine from 9 of 24 (37.5%) patients with LA, but no further details of the patients are given (79).

Of four patients with chronic LA, *B. burgdorferi* DNA was found by PCR with primers specific for the flagellin, *ospB*, and 16S rRNA genes in the CSF of one patient with relapsing

arthritis. This patient had no clinical evidence of CNS involvement and no intrathecal antibody production (101).

Patients with Ocular Manifestations

Ocular manifestations of Lyme disease are rare (95). *B. burgdorferi* DNA could be found in the CSF and urine of a 71-year-old woman with cephalea and a profound decrease in vision (85) and in the CSF and vitreous fluid of a 15-year-old girl who developed pigmentosa-like fundus changes in the left eye as well as a neuropathy in the right eye (82). The serum and CSF of both patients were antibody negative.

Comparison of PCR Results with Serologic Test Results

Specific antibodies can generally be detected only weeks after a tick bite (106). Not surprisingly, in patients with early disease, an inverse correlation between serologic test and PCR results was seen: 88.9% of untreated patients ($n = 90$) with EM were positive by PCR, in contrast to 11 to 16% by IgG ELISA or 34 to 40% by IgM ELISA. The variations are due to different antigens used in the ELISAs (156). Similar results have been reported in other studies (116, 117, 161).

In patients with late disease, the sensitivity of serologic testing is high, but the persistence of antibodies after treatment can cause problems in the diagnosis of patients with ongoing symptoms, and antibody-negative patients have been reported (28, 43, 159).

Comparison of PCR Results with Culture

The sensitivity of culture is lower than that of PCR in all studies. Even when PCR was performed on cultures of skin biopsy specimens from patients with EM, culture was less sensitive than PCR (161). After 2 weeks of culture, PCR was reactive in 19 of 20 patients compared with 14 of 20 patients by microscopic inspection.

No positive cultures have ever been found in CSF samples from patients with late disease (93), and cultures from SF succeeded in only a few patients with LA (123). Therefore, in a patient with a negative culture and a positive PCR result, the likelihood of a false-negative culture is much higher than that of a false-positive PCR result.

With culture, only viable spirochetes can be detected. This is in contrast to PCR, where reactivity can be due to dead organisms, portions of spirochetes that include DNA (blebs), or soluble DNA (84, 105, 123). The PCR results in patients with neuroborreliosis and LA emphasize the improved diagnostic ability available with PCR tests.

Subtyping of *B. burgdorferi* Sensu Lato in Infected Patients

Evidence is growing that the disease manifestations of Lyme borreliosis might be related to different genospecies of *B. burgdorferi* (178). Subtyping of *B. burgdorferi* sensu lato strains in clinical samples from infected patients has been reported by several groups (9, 12, 125, 187). Isolates from skin lesions of patients with ACA were always *B. afzelii*, whereas in all patients with LA, *B. burgdorferi* sensu stricto was found. Patients with EM can harbor all three genospecies of *B. burgdorferi* sensu lato, as analyzed in a German study: 28 of 35 were *B. afzelii*, 6 of 35 were *B. garinii*, and 1 of 35 was *B. burgdorferi* sensu stricto (187). CSF from patients with neuroborreliosis has been found to harbor only *B. afzelii* (9), either *B. afzelii* or *B. burgdorferi* sensu stricto (12), or one of all three genospecies (32). Busch et al. (24a) found all three genospecies of *B. burgdorferi* sensu lato pathogenic for humans in CSF samples from 36 patients with neuroborreliosis: 58% *B. garinii*, 28% *B. afzelii*, and 11% *B. burgdorferi* sensu stricto. Of 10 patients with neuroborreliosis in Belgium, 8 were infected by more than one Lyme disease-associated genospecies (32), and mixed infections seem to be absent in pediatric samples (38).

Monitoring of Treatment Efficacy

At present, there is no test for the eradication of *B. burgdorferi* infection. Serologic assays may remain positive for years after successful resolution of early Lyme borreliosis (42). Early treatment of patients is effective, and follow-up studies by PCR are rare. Four PCR- and culture-positive patients, two of whom had skin rashes only and one of whom had systemic symptoms, responded to a 21-day regimen of oral antimicrobial therapy, and plasma samples tested 70 and 100 days after therapy were negative in a PCR assay (51). In another study, formalin-fixed, paraffin-embedded specimens taken from the affected skin of 8 of 14 patients with EM (57%) were PCR positive before therapy; however, after treatment with minocyclin (14 days), all specimens were PCR negative (118).

A study of 236 urine specimens from 116 patients with uncomplicated EM and 69 specimens from patients with EM accompanied by systemic symptoms showed a PCR sensitivity of 91% in pretreatment samples (155). Six months after therapy, the urine samples of 47 patients who were retested were PCR nonreactive. In another study, in which the same PCR method was used with urine samples, 22 of 24 patients with EM gave positive results before treatment. Immediately after treatment, 11 of 19 patients still yielded positive results; however, only 2 of 16 patients tested after 8 weeks and none tested 20 weeks after were positive by PCR of urine samples (158). The demonstration of *B. burgdorferi* DNA in urine or CSF during or at the end of the treatment course can be explained by (i) the extremely high sensitivity of PCR, which can detect only a few DNA copies; and (ii) the assumption that spirochetes that were protected in sequestered sites became accessible to the antibiotics only late during treatment, thus releasing DNA at that time (47, 86, 115).

An interesting phenomenon is that PCR often becomes reactive only after a few days of treatment. The urine of 6 of 10 untreated, PCR-negative patients with EM became reactive when tested immediately after the end of therapy (156). Similar results were reported for two German patients with EM (105) and for patients with neuroborreliosis (93). In the latter study, the urine samples of only 5 of 10 patients were PCR positive before treatment but when tested 3 and 6 days after onset of therapy, the samples of 9 of 10 patients were PCR positive (93). The enhanced sensitivity of PCR in body fluids shortly after the beginning of treatment can be explained by the increased number of dead bacteria due to the action of antibiotics.

The effect of treatment correlates well with a PCR study of blood donors (79). Three patients with urine positive in PCR were found among 13 seropositive healthy adult blood donors. Two of the PCR-positive patients were treated with doxycyline for 10 days, and the PCR was repeated 4, 6, and 36 weeks after therapy. All PCR tests were negative. The third patient refused therapy and remained PCR positive in tests done during the ensuing 9 months.

Correlation of PCR results with clinical improvement could also be shown in patients with late disease, e.g., patients with neuroborreliosis (59, 75, 84, 88, 93); patients with ACA, morphea, and lichen sclerosus et atrophicus (1, 4, 114, 155, 179); patients with LA (19, 22, 52, 78, 123); and a patient with osteomyelitis (126).

PCR results predicted clinical outcome well in a study of eight patients with neuroborreliosis from whom CSF could be obtained both before and after therapy (84). Patients in whom PCR no longer detected bacterial DNA stabilized or improved; those in whom spirochetal DNA was still evident had symptoms that either persisted or worsened over time. In a Danish study, no specific DNA could be found in urine samples of 10 patients with neuroborreliosis taken 2 to 8.5 months after therapy (93).

B. burgdorferi may survive for years in affected synovium (19, 77). Not surprisingly, in studies of pretreatment SF samples taken from 16 patients with LA, 15 (94%) were PCR reactive (123), as were SF samples taken from 12 patients 2 months to 4 years after short courses of oral antibiotics. In contrast, of 19 patients who received either parenteral antibiotics or long courses of oral antibiotics (doxycycline or amoxicillin), only 7 (37%) had positive test results after treatment. In patients with chronic LA who have PCR-negative results despite multiple courses of antibiotic therapy, the lack of response to antibiotics may suggest that these persons may continue to have arthritis for months or even several years after eradication of viable spirochetes from joint fluids (123).

On the other hand, one should be aware that the importance of PCR in monitoring the efficacy of treatment has not yet been established. Studies of negative PCR results despite ongoing symptoms (84), as well as a persistence of PCR reactivity after treatment have been reported. The opposite phenomenon, a positive PCR result after (adequate) treatment, will be discussed in the next section.

Persistence of PCR Reactivity

It has been well documented that *B. burgdorferi* DNA can be detected in skin specimens taken at the site of a previous erythematous lesion 1 to 6 months (in one patient, 45 months) after disappearance of the lesion (89, 172) and can be found in CSF and SF specimens of patients with late disease more than 10 years after infection (22, 78, 84, 93, 96, 114, 123, 155).

Persistence is generally associated with ongoing symptoms after treatment; however, the presence of DNA detected by PCR does not necessarily prove the presence of clinically active disease (84). One has to differentiate between short-term reactivity after onset of therapy and long-lasting persistence.

At 2 to 4 weeks after treatment with ceftriaxone, PCR results are negative in mice (123). Studies with specimens from humans have shown negative PCR results shortly after treatment of patients with neuroborreliosis (84, 88, 93). Two patients have been reported with PCR reactivity in CSF 14 days after onset of therapy (88). In patients with EM, treatment is generally effective (51, 118), although treatment failures with minocycline have been detected by PCR (99).

Long-lasting PCR reactivity or persistence after adequate antibiotic therapy has been reported in a patient in whom multifocal chorioiditis was first recognized and who then developed arthritis despite a 6-week course of doxycycline treatment (200 mg/day) (61). Therapy with ceftriaxone (14 days) was begun; however, after 2 months free of clinical symptoms, the visual disturbances recurred and ophthalmoscopy showed reactivation of the initial foci of chorioiditis.

In a study of urine specimens from EM patients in whom the erythematous rash was followed by systemic complaints, only 9 of 92 were reactive by PCR 6 months after therapy. Reevaluation of the therapy data, however, has found that in two patients treatment had been interrupted for 2 days and in one patient therapy had to be stopped after 5 days. Five patients could be retreated with ceftriaxone (20 days), and follow-up samples were PCR negative, consistent with clinical improvement (156).

Persistence years after infection has been reported in patients with LA. Six years after an EM rash, which was treated for 2 weeks with oral tetracycline, a patient developed knee pain and swelling and was treated with oral doxycyline for 3 months without improvement. SF showed a leukocyte count of 16.7×10^9 and was positive for *B. burgdorferi* by PCR (22). Among four patients with chronic LA, *B. burgdorferi* DNA was found in the CSF of one patient with relapsing arthritis. This patient had no clinical evidence of CNS involvement and no intrathecal antibody production; this raised the possibility that the CNS acted as a sanctuary for *B. burgdorferi*, protecting it from the action of antibiotics (101).

The persistence of *B. burgdorferi* DNA in patients with LA suggests that persistence of intact organisms or spirochetal components is important in maintaining ongoing immune and inflammatory processes, even among some antibiotic-treated patients. At present, it is not always possible to decide if persistence of *B. burgdorferi* DNA in body fluids is due to a treatment failure, because too little is known about the efficacy of antibiotics in late disease (98, 164).

INTERPRETATION OF PCR RESULTS

What does a positive or a negative PCR test mean? Despite the highest sensitivity available today, a negative PCR result does not exclude the disease. In blood, spirochetemia is transient, and in other body fluids, spirochetes might simply not be present in sufficient numbers at the time samples are taken for analysis. Generally, borreliae, like all spirochetes, seem to be trophic for tissues, avoiding body fluids. They seem to inhabit niches not easily accessable for analysis. However, positive PCR results with CSF of patients with neuroborreliosis, SF of patients with LA, and urine of infected patients emphasize the diagnostic power of PCR tests.

The presence of PCR-detectable sequences does not necessarily prove the presence of an active disease. PCR cannot differentiate between DNA from live and dead organisms. For example, it is not yet known whether whole organisms, components of borreliae, or soluble DNA in urine is the source used for PCR. In SF from patients with untreated LA, plasmid DNA can be detected but genomic DNA is found only rarely. It is not known if whole spirochetes are present in SF or only in membrane vesicles containing extrachromosomal DNA (blebs). Nevertheless, evidence is growing that a positive PCR test can be associated with active disease. After adequate therapy, PCR results are usually negative. Treatment failures associated with ongoing or worsening symptoms are generally also indicated by a persistence of PCR reactivity.

A PCR test must be interpreted in light of knowledge of the clinical context and the limitations of the technique. A positive PCR test obtained from a clinical specimen from a patient who has recently received adequate antibiotic therapy is not proof that further antibiotics are indicated. On the other hand, treatment is probably indicated if a positive PCR result is obtained from a clinical specimen of a patient who has never been treated for Lyme borreliosis (163).

FUTURE DEVELOPMENTS

The diagnosis of Lyme borreliosis is currently based on the presence of a characteristic clinical picture, exposure in an area of endemic infection, and an elevated antibody response to *B. burgdorferi*. However, in situations in which these criteria are

unsatisfactory or inconclusive, new and improved diagnostic methods are needed.

PCR for *B. burgdorferi* infection has been used in animal (10, 17, 24, 65, 92) and vaccine (129, 144) studies. Most studies in humans have been done with only a few patients in specialized laboratories. As can be seen from the number of papers published so far, no standardization of sample preparation, target genes, or PCR and detection methods has yet been established. Parallel evaluations in different laboratories are rare (123); however, they are essential for standardization. Quantitative PCR may be valuable in monitoring treatment response; however, only studies in animals have yet been published (16).

Another important issue is automation. Most procedures have been developed and used in research centers where sophisticated sample extraction, radioactively labelled probes, etc., are readily available. These methods may not be easily adaptable to routine work. Contamination problems do increase dramatically if the number of samples tested daily increases. Although the amplification process itself is simple and multiple target genes (multiplex PCR) can be amplified simultaneously, DNA extraction and preparation from clinical samples before amplification, as well as detection of products and controls of inhibition and contamination after amplification, can take many hours. Several companies have introduced machines in which amplification and detection are done automatically, and robots have been tested with success (188). The next generation of PCR equipment will probably include processors for automatic DNA preparation.

Standardization and automation will increase the number of users of PCR tests and when physicians have been trained to interpret results correctly, the method will open a new dimension in the diagnosis of *B. burgdorferi* infections.

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