Circulating *Borrelia burgdorferi* in Patients with Acute Lyme Disease: Results of Blood Cultures and Serum DNA Analysis

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To determine the usefulness of blood culture and polymerase chain reaction (PCR) analysis in detecting circulating *Borrelia burgdorferi* or its DNA, blood and serum from untreated patients with acute Lyme disease were examined. None of the cultures of blood or serum from the 7 patients tested demonstrated spirochetes. Similarly, all patient serum samples, assayed in two laboratories, were negative for *B. burgdorferi* DNA using PCR amplification. These results suggest that in patients with acute Lyme disease, spirochetes, spirochete DNA, or both circulate early, only intermittently, or at low levels and that neither culture nor PCR testing of blood or serum, as currently done, appears likely to prove generally useful in the diagnosis of Lyme disease.

In the absence of erythema migrans (EM), a skin lesion thought to be characteristic of infection with *Borrelia burgdorferi* [1], it is difficult to make a secure diagnosis of Lyme disease for at least two reasons. First, the clinical manifestations of both acute and chronic Lyme disease are highly variable and not specific [1, 2]. Second, serologic and immunologic testing have not been satisfactorily standardized. Considerable interlaboratory variation and nonspecific reactivity have been documented for virtually all available diagnostic techniques [2–4]. Hence, there has been an on-going effort to determine the usefulness of other potential diagnostic tools.

Two such investigational approaches include direct spirochete isolation from blood or serum [5] and detection of *B. burgdorferi* DNA in clinical samples using polymerase chain reaction (PCR) amplification [6]. Since spirochetemia is thought to occur in the early stages of infection [7, 8], we examined blood and serum from patients with acute Lyme disease to test in a pilot study the potential diagnostic usefulness of both culture and PCR analysis.

Materials and Methods

Serum and heparinized blood were obtained from 7 untreated adults with a clinical diagnosis of acute Lyme disease. Each patient lived in an endemic area (Westchester County, NY), had a compatible flu-like illness with myalgias and fever, and had typical EM skin lesions of 1–10 days duration before venipuncture.

Cultures were established by adding 0.5 mL of blood or serum to 6 mL of modified BSK medium containing 100 μg/mL each of nalidixic acid and 5-fluorouracil [5, 9]. Cultures were incubated at 34°C in 5% CO_2–95% air for up to 12 weeks. At weekly intervals, 15 μL of culture medium was removed and examined by phase-contrast microscopy for the presence of spirochetes. In our hands, phase-contrast microscopy is comparable to darkfield examination for identifying *B. burgdorferi*. A high-passage *B. burgdorferi* strain (ATCC 53899) and a low-passage strain (N40; provided by A. Steere, Tufts University School of Medicine, Boston) were passed weekly in the same BSK medium.

Serum samples were analyzed for *B. burgdorferi* DNA using PCR amplification targeted to a conserved 371-bp chromosomal sequence [6]. PCR was done [6, 10] using an automated DNA thermocycler (Perkin-Elmer Cetus, Norwalk, CT) and 40 cycles. Ten microliters of each completed PCR sample was analyzed on a 2% agarose gel (FMC BioProducts, Rockland, ME) by ethidium bromide staining. To prepare patient samples, 1 mL of serum was centrifuged at 13,000 *g* for 10 min. The pellet was resuspended in 10 μL of TE (10 m*M* TRIS, pH 7.4, 1 m*M* EDTA), boiled for 5 min, and then amplified by PCR [6, 10]. For positive control reactions, washed strain 53899 or N40 spirochetes were resuspended in NTE (150 m*M* NaCl, 10 m*M* TRIS, pH 7.4, 1 m*M* EDTA), counted in a hemocytometer, and either added directly to the PCR reaction mixture or diluted in buffer or seronegative control human serum to 1 spirochete per milliliter using serial 10-fold dilutions. These buffer and serum samples were then centrifuged and processed for PCR analysis as described above.

Southern blotting was done on amplified DNA using an internal probe end-labeled with (γ-^{32}P) ATP. DNA was transferred to nylon membrane by capillary method [11] or by semidry electrophoresis (Trans-Blot; Bio-Rad, Richmond, CA). Membranes were UV-cross-linked, hybridized with the radiolabeled probe, and exposed to Eastman Kodak (Rochester, NY) XAR film at −70°C with an intensifying screen.

Groups of 5 or 6 20- to 30-g female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were injected intravenously by

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tail vein with 10^7 strain 53899 or N40 spirochetes. Serum for PCR analysis was obtained 5 min after injection.

Results

Cultures of blood and serum obtained from the 7 patients with EM were maintained for 12 weeks. None of these 14 cultures demonstrated spirochetes. Using the same medium, culture conditions, and dilutions made in buffer or serum, strains N40 and 53899 grew readily to concentrations of up to 10^8/6.5 mL of culture medium 1 week after inoculation with 10^5 spirochetes. For strain N40, the mean (±SE) doubling time was 14.6 ± 0.6 h during the first week of cultivation (4 experiments, not shown).

To determine the lower limit of spirochete detectability in this culture system, N40 organisms were serially diluted in control serum, and 0.5 mL of serum (calculated to contain 10^5 spirochetes) was added to 6 mL of culture medium. After 1 h at 34°C, duplicate 15-μL aliquots were removed and examined. By phase-contrast and darkfield microscopy, we could detect spirochetes in cultures inoculated with 10^4 organisms per 6.5 mL (e.g., 1.5×10^3/mL) (two experiments). Together with the finding that 2 laboratory strains replicated well under the same culture conditions, we believe our system was sufficiently sensitive to detect spirochetes in our patients' long-term cultures.

Although not done at the time our patients' samples were cultured, we subsequently found that centrifuging the culture medium could enhance spirochete detection. One hour after inoculating 6 mL of culture with 0.5 mL of serum containing 10 to 1×10^3 organisms, duplicate 1-mL aliquots were removed and centrifuged at 13,000 *g* for 10 min; the pellet was resuspended in 50 μL of medium. Using this technique, spirochetes were detected microscopically in cultures inoculated with as few as 10 organisms (two experiments). This manipulation as well as other more recently reported culture techniques [12] may prove useful in future studies.

PCR analysis of sera from the 7 EM patients tested also failed to show positive results for *B. burgdorferi* DNA. In 2 experiments, however, the PCR technique used was able to detect the presence of 1 culture-derived spirochete when added directly to the reaction mixture (figure 1). Of interest, PCR sensitivity declined if spirochetes had been exposed to serum. In two experiments, the lower limit of PCR detectability was 100 spirochetes for organisms obtained from pellets of serial 10-fold dilutions made in control serum. In the same experiments, the PCR assay detected the presence of ≤10 spirochetes, which had first been diluted in buffer (data not shown).

To validate our negative PCR results, two approaches were used. First, serum samples from the 7 patients, sera from 5 healthy volunteers, and buffer inoculated with spirochetes were assayed in another laboratory (Yale University School of Medicine) where PCR testing for *B. burgdorferi*

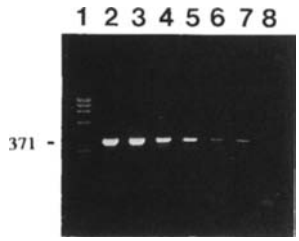

Figure 1. Polymerase chain reaction (PCR) amplification of *B. burgdorferi* DNA. Ethidium-stained PCR products separated by gel electrophoresis. DNA size (371 bp) is shown at left. Lanes: 1, DNA size marker; 2–5, 10^5, 10^4, 10^3, and 10^2 spirochetes, respectively; 6, 10 spirochetes; 7, 1 spirochete; and 8, no spirochetes. All samples were added directly to 50 μL of PCR reaction mixture. Southern blotting with radiolabeled internal probe was done to verify identity of PCR products (not shown).

DNA [10] has been positive using human synovial fluid in Lyme arthritis [13]. This independent testing, using DNA directly extracted from 0.1 mL of serum, outer surface protein A primers 6 and 7 [13], and conditions under which ≤10 copies of *B. burgdorferi* DNA can be detected, confirmed our negative serum PCR results. Among all the samples, only the control specimen in buffer was positive.

Second, BALB/c mice were injected intravenously with 10^7 spirochetes. In 2 experiments, 5 min after injection of strain 53899 or strain N40, sera from 5 of 11 and 3 of 6 mice, respectively, were positive by PCR analysis (data not shown). Thus, while positive in only 50% of animals, these results indicated that the PCR technique under controlled conditions could detect *B. burgdorferi* DNA in the circulation.

Discussion

The results of this pilot study suggest that neither culturing blood or serum nor PCR analysis of serum is likely to provide much diagnostic yield in patients with Lyme disease. We studied patients with acute infection, reasoning that, if sensitive, these techniques should be positive in those most likely to be spirochetemic. Our culture results contrast with data from a previous study in which blood cultures from 4 (21%) of 19 patients with EM demonstrated spirochetes [5]. However, our findings are consistent with prior reports that showed *B. burgdorferi* is seldom isolated from either blood or serum [7, 8, 14].

Our observations in mice experimentally infected with a large inoculum suggest that *B. burgdorferi* spirochete DNA can be detected in serum by PCR analysis; other investigators have also reported a correlation between culture and PCR results using ear biopsies from infected mice [10]. Thus, PCR testing may prove useful in the tissue diagnosis of Lyme disease. The number of organisms likely to be present in a serum specimen from an infected patient, however, is likely to be well below that induced in our infected mice. In addi-

tion, blood specimens themselves may also contain inhibitors of PCR [15, 16], which may account for the decrease in PCR sensitivity we observed using serum- versus buffer-treated spirochetes. Thus, samples such as serum or tissue contaminated with blood or factors in blood [16] may also be intrinsically less suitable for diagnostic PCR testing for Lyme disease, at least until specimens can be appropriately concentrated and systematically freed of potential inhibitors.

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