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Comparison of Five Diagnostic Modalities for Direct Detection of *Borrelia burgdorferi* in Patients with Early Lyme Disease

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Abstract

Lyme disease, the most commonly reported tick-borne infection in North America, is caused by infection with the spirochete *Borrelia burgdorferi*. Although an accurate clinical diagnosis can often be made based on the presence of erythema migrans, in research studies microbiologic or molecular microbiologic confirmation of the diagnosis may be required. In this study, we evaluated the sensitivity of five direct diagnostic methods (culture and nested PCR of a 2 mm skin biopsy specimen, nested PCR and quantitative PCR (qPCR) performed on the same 1 mL aliquot of plasma and a novel qPCR-blood culture method) in 66 untreated adult patients with erythema migrans. One or more these tests were positive in 93.9% of the patients. Culture was more sensitive than PCR for both skin and blood, but the difference was only statistically significant for blood samples ($p< 0.005$). Blood culture was significantly more likely to be positive in patients with multiple erythema migrans skin lesions compared to those with a single lesion ($p=0.001$). Positive test results among the 48 patients for whom all five assays were performed invariably included either a positive blood or skin culture.

The results of this study demonstrate that direct detection methods such as PCR and culture are highly sensitive in untreated adult patients with erythema migrans. This enabled microbiologic or molecular microbiologic confirmation of the diagnosis of *B. burgdorferi* infection in all but four (6.1%) of the 66 patients evaluated.

Lyme disease, caused by *Borrelia burgdorferi*, is the most commonly reported tick-borne infection in North America (Bacon *et al.* 2008). Erythema migrans (EM) is the most common clinical manifestation (Dandache and Nadelman 2008). For most patients with EM-like skin lesions in clinical practice the diagnosis can be made by visual inspection. However, in research studies, microbiologic or molecular microbiologic confirmation of the diagnosis may be required and a number of diagnostic modalities have been used successfully in this setting including polymerase chain reaction (PCR) and culture of both skin biopsy specimens and blood (Aguero-Rosenfeld *et al.* 2005; Wang *et al.* 2010).

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We have recently reported that the sensitivity of blood cultures in patients with EM can be significantly improved by testing aliquots of culture medium after short time periods of incubation for the presence of *B. burgdorferi* DNA by a quantitative PCR (qPCR) method (hereafter called qPCR-blood culture) (Liveris *et al.* 2011a). In addition, we have optimized the sensitivity of PCR on blood specimens directly by sampling a 1 mL aliquot of plasma and by using both qPCR and nested PCR (nPCR) assays (Liveris *et al.* 2011b).

In this study, we compare the sensitivity of five diagnostic modalities in untreated adult patients with EM.

Methods

Patients

Plasma samples in EDTA-blood collection tubes were obtained from adult patients with a clinical diagnosis of EM that had been established at the Lyme Disease Diagnostic Center (Valhalla, NY) in the years 2005, 2006 and 2007. All eligible patients with EM were invited to participate in this study. Samples were excluded from this analysis if the patients had recently been treated with a beta-lactam or tetracycline antibiotic before the plasma sample was obtained. The protocol was approved by the Institutional Review Board of New York Medical College and written informed consent was obtained from all participants.

Blood cultures using quantitative PCR on culture aliquots

Cultivation of 9 mL plasma specimens was performed as described previously (Liveris *et al.* 2011a). Briefly, (three 3-mL aliquots of plasma were inoculated into separate flasks containing 60 mL of antibiotic-free Barbour-Stoenner-Kelly (BSK) medium. Cultures were incubated at 32°C – 33°C for 8–12 weeks (12 weeks in 2005 and 2006 and 8 weeks in 2007), and were examined for spirochetes by fluorescence microscopy at 2 weeks and thereafter at 2–4 week intervals. A 10-μL aliquot of culture material was mixed with 10 μL of an acridine orange staining solution (100 μL in PBS [pH 7.41]), placed on a slide, overlaid with a coverslip and examined at 400x magnification. A minimum of 20 high-power fields were viewed for the presence of spirochetes. Isolation of borrelial DNA from culture aliquots after the cultures had been incubated for time periods ranging from 1 day to 21 days and application of real time qPCR to this DNA, were performed as described previously (Liveris *et al.* 2011a).

Skin biopsy and culture

Skin biopsy specimens (2 mm in diameter) were obtained from the advancing border of primary EM lesions, as described elsewhere (Nowakowski *et al.* 2001). Biopsy specimens were placed into transport medium, which consisted of modified BSK medium (this preparation of BSK lacks rabbit serum and bovine serum albumin) plus rifampin 40 μg/mL, for later laboratory processing. Tissue specimens were then transferred to a micro tissue grinder (Spectrum Medical Industries), which contained 0.4 mL of modified BSK medium without rifampin, and were ground; 0.2–0.3 mL of this suspension was added to a 7-mL screw-cap tube that contained 6 mL of complete BSK medium (with rabbit serum and 35% BSA solution, but devoid of antibiotics). The screw-cap tube was tightly capped and incubated at 33°C for the duration of the culturing period. The remaining suspension plus the skin fragment itself were returned to the transport medium and sent for PCR studies. Cultures were examined by means of fluorescence microscopy at 2 weeks and, thereafter, at 2-week intervals for up to 8 weeks, as previously reported (Nowakowski *et al.* 2001).

PCR of plasma aliquots

Plasma was separated from whole blood by centrifugation at 260Xg for 15 minutes. A 1 mL aliquot of plasma was then centrifuged at 14,000Xg for 10 minutes, pellets were resuspended in 100 μL of IsoQuick sample buffer, DNA was extracted by means of a commercial nucleic acid extraction kit (IsoQuick; Orca Research, Bothell, WA) as described (Schwartz *et al.* 1993) and suspended in 50 μL of distilled water, RNA grade (Fisher Scientific). Two microliters of DNA suspension was used for PCR amplification. Nested PCR was performed as previously described (Liveris et al., 2002; Barbour et al. 1996). Real-time qPCR was performed in an ABI Prism 7900HT Sequence Detection System (Taqman) using two *flaB*-specific primers FL-571F and FL-662R, as described (Liveris *et al.* 2011b).

Statistics

Categorical variables were compared by the Fisher's exact test (two-tailed). A p value <0.05 was considered to be significant.

Results

The 66 patients in this study were adults between the ages of 19 and 84 years, 36 (54.5%) of whom were male. Fifty-two (78.8%) of the 66 patients had all five direct diagnostic tests performed namely culture and nPCR of a 2 mm skin biopsy specimen, nPCR and qPCR done on the same 1 mL aliquot of plasma and qPCR-blood culture. Some of the results have been previously reported (Liveris *et al.* 2011a; Liveris *et al.* 2011B).

Culture was more sensitive than PCR for both skin and plasma, but the difference was only statistically significant for plasma using the qPCR-blood culture method ($p< 0.005$). It should be noted that the conventional culture method was used for skin biopsy samples in which a culture was determined to be positive solely based on microscopic detection of spirochetes in culture medium after periods of incubation of up to 8 weeks (Table 1). PCR and culture were both more sensitive on plasma samples of patients with multiple skin lesions compared to those with a single lesion. A statistically significant difference was observed, however, for only the comparison of qPCR-blood culture of patients with multiple EM skin lesion compared to patients with a single lesion (22/23 with multiple EM skin lesions were positive versus 24/42 with a single EM skin lesion, $p=0.001$). Only four patients (6.1%) were negative on all of the tests that were performed, three of whom had a single EM skin lesion. Of the 62 patients (93.9%) with at least one positive test result, all but 7 (11.3%) had positive test results on more than one of the tests performed. For six of the seven with just a single positive test all five assays were performed.

Fifty-two patients had all five assays performed, 48 (92.3%) of whom had at least one positive test result (Table 2). Positive test results among these 48 patients invariably included either a positive qPCR-blood culture or a positive skin culture. Although it could be argued that the 4 patients with negative assays were misdiagnosed and did not actually have active *B. burgdorferi* infection, this would seem unlikely, since 3 were found to be seropositive for antibodies to *B. burgdorferi* on acute or convalescent phase testing (data not shown).

Discussion

Numerous published studies have reported on the utility of culture or PCR for detection of *B. burgdorferi* in clinical specimens (Aguero-Rosenfeld *et al.* 2005; Wang *et al.* 2010). In general, cultivation of skin biopsies obtained from EM lesions of untreated patients resulted in >50% positivity; the highest reported culture yield in US patients was 86% in a study that employed 4 mm skin biopsies (Berger *et al.* 1992). In two earlier published series from our

group, culture of 2 mm skin biopsies was positive in 57% (Schwartz *et al.* 1992) and 54% (Liveris *et al.* 2002) of patients, consistent with the 61.5% yield obtained in the present study. PCR of skin biopsy specimens has typically resulted in a higher positivity rate than culture; the median sensitivity in 17 published studies was 69% (Wang *et al.* 2010). We have previously reported positive PCR results in 59% and 64% of skin biopsy specimens (Schwartz *et al.* 1992; Liveris *et al.* 2002), a rate essentially identical to that obtained with culture. In the current study, only 42.6% of skin biopsy specimens were positive by nested PCR. The reason for this discrepancy is not clear, but is not likely to be due to PCR inhibitors present in the processed specimens, as the procedures were identical to those employed in our earlier studies that yielded the higher PCR positive rates. Furthermore, samples were processed within 12–24 hours of collection and were kept refrigerated until processing, making degradation of DNA unlikely.

Twenty-nine of 65 (44.6%) plasma samples were positive by a PCR method (either nested or quantitative PCR). This is similar to a previous report by Jones et al. (Jones *et al.* 2006) and higher than that reported in several other studies (Aguero-Rosenfeld *et al.* 2005; Goodman *et al.* 1995). This value is also consistent with the positivity rate obtained by culture using the conventional end-point of microscopic detection of spirochetes in the culture medium (Nowakowski *et al.* 2001). Of note, the novel qPCR-blood culture technique used in this study, in which a positive culture was determined based on a positive qPCR assay of culture aliquots rather than microscopic detection, resulted in 70.8% rate of positive blood cultures overall, which increased to 95.7% (22/23) in the subgroup of patients with multiple EM skin lesions.

Direct detection methods such as PCR and culture have the advantage of high specificity and can assist in classifying and characterizing the strains of *B. burgdorferi* causing the infection. As shown here and as in discussed in more detail elsewhere (Liveris *et al.* 2011a), the qPCR-culture method of plasma was able to identify evidence of borrelial DNA in plasma of all but one of the patients with multiple EM skin lesions further reinforcing the concept that secondary EM skin lesions arise by hematogenous dissemination.

The results of this study demonstrate that direct detection methods such as PCR and culture are highly sensitive in untreated adult patients with EM allowing microbiologic or molecular microbiologic confirmation of the diagnosis of *B. burgdorferi* infection in 94% of the 66 patients evaluated. The most efficient way to identify *B. burgdorferi* infected patients was through the use of the qPCR-blood culture of plasma in combination with skin culture.

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Table 1

Comparison of PCR and Culture Tests in Adult Patients with EM

Sample Tested	**Method of Testing**				**P value**
All EM Patients n=66					
	nPCR	Standard culture			
Skin	23/54 * (42.6%)	34/55 (61.8%)			0.06
	nPCR	qPCR	Either nPCR or qPCR	qPCR-blood culture	
Plasma	26/64 (40.6%)	22/65 (33.8%)	29/65 (44.6%)	46/65 (70.8%)	0.004 **
Multiple EM Patients n=23					
	nPCR		Standard culture		
Skin	7/19 (36.8%)		9/20 (45.0%)		0.75
	nPCR	qPCR	Either nPCR or qPCR	qPCR-blood culture	
Plasma	11/23 (47.8%)	9/23 (39.1%)	14/23 (60.9%)	22/23 (95.7%)	0.01 **
Single EM Patients n=43					
	nPCR		Standard culture		
Skin	16/35 (45.7%)		25/35 (71.4%)		0.051
	nPCR	qPCR	Either nPCR or qPCR	qPCR-blood culture	
Plasma	15/41 (36.6%)	13/42 (31.0%)	15/42 (35.7%)	24/42 (57.1%)	0.08 **

* Number positive/number tested

** For the comparison of the frequency of a positive qPCR-blood culture with the frequency of a positive test result for either nPCR or qPCR directly on plasma

Table 2

Frequency of Positive Test Results for the 52 Patients with EM for Whom All Five Assays were Performed.

Positive on at least one assay	48 (92.3%)
Positive qPCR-blood culture	39 (75.0%)
Positive nPCR plasma	20 (38.5%)
Positive qPCR plasma	16 (30.8%)
Positive nPCR of skin	22 (42.3%)
Positive culture of skin	32 (61.5%)
Positive qPCR-blood culture or positive skin culture	48 (92.3%)
Positive on exactly 5 assays	4 (7.7%)
Positive on exactly 4 assays	8 (15.4%)
Positive on exactly 3 assays	13 (25.0%)
Positive on exactly 2 assays	15 (28.8%)
Positive on exactly 1 assay	8 (15.4%)