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INVESTIGATIVE REPORT

Detection of Spirochaetal DNA Simultaneously in Skin Biopsies, Peripheral Blood and Urine from Patients with Erythema Migrans

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Lyme borreliosis is an emerging zoonosis transmitted by infected hard-bodied ticks. The disease is multisystemic. In the initial stage its typical manifestation is the erythema migrans, a cutaneous lesion that occurs in up to 90% of patients. In order to investigate the presence of the specific agent, *Borrelia burgdorferi*, in the early stages of the disease, DNA from skin biopsies, urine and peripheral blood of 30 patients with clinically documented erythema migrans and without apparent systemic involvement was analysed by polymerase chain reaction. *Borrelia* DNA in both blood and skin biopsies was detected in 23 patients, while in 9 patients it was discovered in urine and skin biopsies. These results demonstrate that *Borrelia* DNA is detectable systemically also in patients with early Lyme borreliosis and strongly suggest a possible dissemination of the causative agents even when only a local infection is assumed. ***Key words: erythema migrans; Lyme borreliosis; paraffin-embedded tissues; PCR analysis.***

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Lyme borreliosis (LB) is an emerging tick-borne spirochetosis transmitted by the bite of infected hard-bodied ticks of the genus *Ixodes*. In Europe, the principal vector of *Borrelia burgdorferi* is *Ixodes ricinus*.

LB is a multisystemic disease involving skin, joints and the nervous system. The typical cutaneous manifestation of the primary stage is erythema migrans (EM), an expanding red or bluish-red rash with central clearing. This lesion occurs in up to 90% of patients with objective evidence of LB (1). Discordant results have been reported about the clinical manifestation of LB and the different species of infecting *Borrelia*. No differences between the occurrence of EM and infection with specific *Borrelia* species have been detected by some authors (1), while others point out that strains of *B. afzelii* have been found mainly in cutaneous manifestations of the disease, such as EM, and especially in acrodermatitis chronica atrophicans (2). However, EM appears at the early stage of *B. burgdorferi* infection, persisting from a few days to several weeks after the bite of the infected tick, and the lesion can be associated with systemic manifestations in about 20% of cases (2, 3).

The progression of the disease is not fully understood. The entry of the *Borrelia* infection is assumed to be through the skin. However, the first stage of the disease may lack cutaneous manifestations. There is a local reaction at the bite site and subsequently the blood carries the pathogens to other organs. A complex inflammatory reaction includes plasminogen and complement activation, phagocytosis of *Borrelia* by local macrophages and presentation of the lysed organisms to the immune system, which will allow a specific immune response. At the same time, activated macrophages produce proinflammatory substances such as monokines and chemokines that induce vessel dilatation, increase vascular permeability, diapedesis of granulocytes and monocytes and chemotaxis of these cells. When the non-specific cellular and humoral defences are unable to eliminate the spirochetes the disease tends to progress (4).

The diagnosis of LB is primarily clinical, but serological tests can provide useful supporting evidence. ELISA and Western blot assays are the most widely used, but, despite continuous improvements, they still present a low specificity (1). Cross-reactive antibodies can produce false-positive results in patients affected by other bacterial or viral infections, autoimmune and rheumatic diseases. False-negative reactions are common in the early stages of LB or in immunosuppressed patients. Cultivation of *B. burgdorferi* from body fluids is slow and inefficient, indicating the need for new diagnostic tools (5). The polymerase chain reaction (PCR) is a sensitive method for the diagnosis of microorganisms that are difficult to cultivate (5). This analytical method allows direct detection of very few genomes of *B. burgdorferi* in different clinical specimens (6), including skin biopsies, urine, peripheral blood, synovial fluid and cerebrospinal fluid. To investigate *B. burgdorferi* spreading during the first

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stages of the disease with skin local infection, we analysed skin biopsies, urine and peripheral blood from 30 patients with clinically documented EM.

MATERIALS AND METHODS

Patients

Thirty patients with EM (17 females and 13 males) were examined in the Dermatology Department of the University of Trieste, Italy from January 2000 to January 2002. Their average age was 48.4 years, ranging from 23 to 87 years; the median age was 48.0 years (25th to 75th percentile = 30.5 – 61.5).

All patients reported a tick bite 1 to 4 weeks before dermatological examination during recreation activities in the country around the city of Trieste, which is an endemic region in north-eastern Italy. All patients were clinically diagnosed as early stage skin LB on the basis of EM by experienced dermatologists (MGI, PP or GT). A careful history focusing on signs and/or symptoms suggestive of systemic infection was taken for each patient. All skin lesions lasted less than 4 weeks and none had received antibiotic therapy prior to clinical diagnosis and at least one month before the tick bite.

All patients were treated for 14 days with 1 g of Amoxycillin three times daily in accordance with recommended guidelines.

The exclusion criteria for patients in this case study were: (i) diagnosis of LB in the past, (ii) patients who reported other tick bites in the past; (iii) patients who could not remember having been bitten recently by a tick; (iv) patients with EM who reported concomitant symptoms or clinical signs of general infection; (v) the use of any systemic antibiotic in the preceeding 2 months; (vi) previous diagnosis of autoimmune or rheumatic diseases; (vii) active infections of other pathogens; and (viii) immunosuppressed patients.

Samples

Skin biopsies. Six-millimetre skin biopsies from patients were taken from the margin of the primary EM lesion. Biopsy specimens were formalin-fixed and paraffin-embedded for histological examination, and subsequently 10 histological sections were submitted for PCR analyses.

Serology. Sera were examined for *B. burgdorferi* IgM and IgG antibodies by ELISA (Lyme Borreliosis ELISA kit IgG/IgM, Dako, CA, USA) in accordance with the manufacturer's instructions. The ELISA analysis was performed in each patient before and after specific therapy.

DNA preparations

DNA extractions, amplifications and post-amplification procedures were performed in accordance with the precautions suggested by Kwok & Higuchi (7).

DNA preparation from paraffin-embedded biopsies. DNA was extracted from 10-μm sections of paraffin-embedded blocks. Ten sections were cut from every sample with standard microtomes. The blade was shifted after each block to prevent cross-contamination between samples. As previously reported (8), paraffin was removed by two washes with xylene, followed by two washes in ethanol, 100% and 70%. After air-drying, the tissue pellets were digested overnight at 45°C with proteinase K (0.5 μg/ml) in 50 mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20. To purify DNA from proteinase K and proteolysis residues, an extraction with phenol-Tris/chloroform was performed. The final concentration of DNA from the solution was made by precipitation with ethanol using 5 μl of glycogen 1 mg/ml as precipitation carrier.

DNA preparation from blood samples. Five millilitres of fresh blood from each patient was collected in an EDTA tube and submitted to DNA extraction. Forty-five millilitres of lysis buffer containing 0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ and 1% Triton X 100 was added to each blood sample. The samples were mixed by inversion several times, incubated at 4°C for 10 min and then centrifuged at 600 × g for 15 min at 4°C. Supernatants were decanted, the pellets were washed in 5 ml of cold PBS and centrifuged as reported above. The resulting pellets were resuspended in 1 ml of lysis solution containing 10 mM Tris-HCl pH 8, 400 mM NaCl, 2 mM EDTA, 0.7% SDS, 1 mg/ml of Proteinase K. Digestion was left to proceed at 45°C for at least 4 h under gentle shaking. To precipitate protein debris, one-third of total volume of NaCl saturated solution was added to samples. Tubes were vigorously mixed for 10 sec and then centrifuged at maximum speed (13000 × g) for 5 min at room temperature. Supernatants were collected and DNAs were precipitated with one volume of iso-propanol at room temperature. Genomic DNAs were picked, washed with 0.5 ml of 70% ethanol, air dried and resuspended in 100 μl of sterile water.

DNA preparation from urine samples. For the extraction of DNA from urine samples the alkaline lysis method (9) was avoided and a protein digestion procedure was chosen in order to eliminate PCR protein inhibitors. Urine samples (20 ml each) were centrifuged at 600 × g for 15 min at 7°C. The sediments were washed with 5 ml of PBS and sedimented by another centrifugation step. Urine residues were mixed with 10 volumes of digestion solution composed of 10 mM Tris pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.4% SDS and 1 mg/ml proteinase K (10). Samples were incubated at 45°C overnight. To eliminate protein debris an extraction with phenol-Tris/Chloroform was performed. DNAs were isolated by iso-propanol precipitation.

PCR amplification

We avoided nested PCR because of the high risk of carry-over connected with this method. In order to get a higher sensitivity, especially in paraffin-embedded tissues where DNA is highly degraded (11), we decreased the amplicon size to a maximum length of 100 bases and increased the number of PCR cycles to 70. Every PCR reaction was run in duplicate. Primer sets were chosen in regions of the Borrelia chromosome with low variability. For the analysis, we chose a combination of three primer sets in order to achieve high sensitivity (5). One set of primers targeted a sequence of a chromosomal gene encoding for a 66 kDa protein, the second set targeted the Borrelia flagellin gene (41 kDa protein) and the third primers set was specific for the gene encoding the 80 kDa antigen. Primer sequences are reported below: 66 kDa (GenBank M60802, AE001174); Primer up: 5′-TGCAAA TGGGAACTGATT-3′; Primer dw: 5′-TGAGGGTGTTTCT TTTTT-3′; Probe: 5′-TGGACACATCTCAAAAGCAGCG AA-3′; 80 kDa (GenBank M60802, AE001161); Primer up: 5′-GGTAAAGCCTTGGATCTTGA-3′; Primer dw: 5′-CTTC TTCCTTGGCTTTACTT-3′; Probe: 5′-CGAGAATTAAA TTCTAAAGCTTCTAGC-3′; Flagellin (GenBank AF244889);

Primer up: 5′-TTCTCTGGTGAGGGAGCTCAAAC-3′; Primer dw: 5′-CTGTTGAGCTCCTTCCTGTTG-3′; Probe: 5′-TCA GGCTGCACCGGTTCAAGAGGGTGTT-3′. For every *B. burgdorferi* gene, three oligonucleotides were synthesized, two in DNA sense and one in antisense orientation. The first sense and the antisense oligonucleotides were used for the amplification reaction. The second sense oligonucleotide was used as internal probe for the amplified product in order to detect only specific amplicons. In this way a further test of specificity was performed. To evaluate the sensitivity of the primers sets in blood, urine and paraffin-embedded biopsy tissues (PET), 0.01 pg, 1 pg, 10 pg and 100 pg of Borrelia DNA were diluted in 1 μg of DNA derived from PET (free from LB) and in 2 μg of DNA derived from blood and urine derived from a disease-free donor. Every test was run in duplicate. Positivity was detected for every sample and every primer set. To assess the sensitivity of the different primer sets, serial dilutions of specific Borrelia DNA were performed. With the proposed sets of primers 0.01 – 0.02 pg of Borrelia DNA was detected, corresponding to 5 – 10 Borrelia genomes. The efficiency of the primers was initially evaluated using PET from patients clinically positive for LB. Borrelia DNA was detected in 97% of the cases. In order to assess the specificity of the three primer sets, PET of different microbial skin lesions were analysed. DNAs were obtained from 2 samples of cutaneous tuberculosis and 2 scraps of primary syphilis lesion. Specificity tests were also done using DNA of Mycobacterium avium and Candida albicans (generously provided by the bacteriology unit of ICGEB) and E. coli. In none of the samples was Borrelia DNA detected utilising the proposed analytical method.

The PCR mixture (total volume 50 μl) contained the isolated DNA, 15 pmol of each primer, 200 μM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.0 mM $MgCl_2$ and 1.25 U of TaqPolymerase (Amersham Biosciences, Uppsala, Sweden). Amplifications were carried out for 70 cycles as follows: 1 denaturation step at 94°C for 3 min; 5 cycles of 94°C/1 min, annealing temperature/1 min, 72°C/1 min and 65 cycles of 94°C/30 sec, annealing temperature/30 sec, 72°C/30 sec. The annealing temperature for 66 kDa was 42°C, for 80 kDa 50°C and for Flagellin 55°C. Two micrograms of DNA obtained for blood and urine samples was submitted to PCR amplification and 1 μg of DNA obtained from PET. For every PCR analysis negative controls containing DNA obtained from healthy donors were included. In addition, pure genomic *B. burgdorferi* DNA was used as positive control. For positive controls, 5 ng of specific DNA obtained from *B. garinii, afzelii* and *sensu stricto* were used.

Dot-blot hybridization

The amplifications were tested by dot blot with specific internal probe hybridization. Twenty microlitres of amplified material were denatured for 10 min at 95°C and chilled on ice. After this step, 30 μl of SSC (saline sodium citrate) 20× and 1 μl of dye for dot blot (0.25% bromophenol blue, 2.5% ficoll in sterile water) were added to each sample. Specimens were spotted on a pre-equilibrated Hybond N+ membrane (Amersham Biosciences) using a dot blot apparatus. The membrane was air-dried and cross-linked twice in UV-Stratalinker (Stratagene, La Jolla, CA, USA). In order to overcome the detection of unspecific products, the third oligonucleotide, internal to the amplification fragment, was used as a probe after a kinasation step. Reaction was performed with 500 ng of oligonucleotide using 10 units of T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA, USA) and 50 μCi of [γ-^{32}P]ATP (Amersham Biosciences) for 1 h at 37°C. Labelled probe was then purified onto a G-25 Sephadex (Amersham Biosciences) minicolumn.

After pre-hybridization for 1 h the membranes were hybridized overnight at the proper temperature in SSC 6×, 0.25% milk powder. The hybridization temperatures were 47°C for 66 kDa, 42°C for 80 kDa and 50°C for flagellin. After hybridization, 2 washes in 6× SSC, 0.1% SDS at room temperature, 2 washes in 3× SSC, 0.1% SDS and 2 in 1× SSC, 0.1% SDS at 10°C more than the hybridization temperature were performed. Membrane positivity was detected using a Cyclon Storage Phosphor System (Packard Instrument, Meriden, CT, USA).

Statistical analysis

The chi-square test was performed to assess the differences in the results obtained using the three PCR systems. Statistical analyses were performed using the EPI6 software dedicated to epidemiology studies.

RESULTS

We examined 30 patients with early LB with clinically defined EM. The serological tests with the ELISA method gave positive results for specific IgM antibodies in 8 patients. A control serological examination carried out 2 months after the end of therapy showed sero-conversion with specific IgG antibodies in 3 patients only.

For every patient, the biological specimens were investigated in duplicate by PCR amplification. Blood, urine and skin (PET) were analysed with the three different PCR primer sets specific for different sequences of the *B. burgdorferi* genome (B-80, 66 kDa protein, flagellin). PCR results for each patient are reported in Table I. The percentage of positive results for B-80 in blood was 47%, in urine 10% and in PET 50% (Table II). For 66 kDa protein the rate of positive results was 53% in blood, 20% in urine and only 37% in PET. The third set, detecting the *B. burgdorferi* flagellin gene, gave the best results in archive biopsies (PET) with 86% positivity, 37% in blood and 13% in urine (Table II). Complete concordance of the three sets was detected in 13% of blood samples (4/30), in no urine sample and in 6% (2/30) of PET. *B. burgdorferi* DNA could be detected by at least one of the three sets in 76% (23/30) of blood samples, in 30% of urine samples (9/30) and in 100% (30/30) of PET. No *B. burgdorferi* DNA was detectable in control patients related to dermatological diseases different from LB.

Discordant results were observed for the three sets of primers with statistically significant differences: in PET the flagellin primer set was the more sensitive ($\chi^2 = 14.12$, d.f. $= 2$, $p < 0.001$), whereas no differences were detected in blood ($\chi^2 = 0.82$, $p = 0.66$) and urine samples ($\chi^2 = 1.26$, $p = 0.53$).

The PCR analyses indicated that 7 patients were positive in all 3 biological specimens examined (blood, urine and PET), 16 had positive results in PET and

Table I. *Individual PRC result for Borellia DNA positivity in blood, urine and paraffin-embedded tissue (PET) of 30 patients with erythema migrans using 3 different markers.*

	B-80			66 kDa			41 kDa (Flagellin)		
Pat. no.	Blood	Urine	PET	Blood	Urine	PET	Blood	Urine	PET
1	−	−	−	−	−	+	−	+	+
2	−	−	−	−	−	−	−	−	+
3	+	+	−	+	+	+	−	−	+
4	−	−	−	−	−	+	+	−	+
5	−	−	−	−	−	+	−	−	+
6	+	−	+	+	−	−	+	+	+
7	+	−	+	+	+	+	−	−	+
8	+	−	+	+	−	−	+	−	−
9	−	−	+	+	−	+	−	−	−
10	+	−	+	+	+	+	+	+	−
11	−	−	−	−	−	−	−	−	+
12	+	−	−	−	−	−	−	−	+
13	−	−	−	+	−	−	+	−	+
14	+	−	−	+	−	+	+	−	+
15	−	−	−	+	−	+	−	−	+
16	−	+	+	+	−	+	+	+	+
17	−	−	−	+	−	−	+	−	+
18	+	−	−	+	−	−	−	−	+
19	−	−	+	−	+	−	−	−	+
20	+	−	+	+	−	−	−	−	−
21	+	−	+	−	+	−	−	−	+
22	+	−	+	−	−	−	−	−	+
23	+	−	+	+	−	−	−	−	+
24	−	−	−	−	−	−	+	−	+
25	−	−	−	+	−	−	−	−	+
26	−	−	+	−	−	+	−	−	+
27	−	−	−	−	−	−	−	−	+
28	+	+	+	−	+	−	+	−	+
29	+	−	+	−	−	−	−	−	+
30	−	−	+	+	−	−	+	−	+

blood, 2 in urine and PET and 5 had positive results only in PET. No one was completely negative.

DISCUSSION

In this study, 30 patients with early LB, characterized by the presence of EM, were investigated. Three biological specimens were analysed for each patient: skin (PET), blood and urine. Patients with EM, but without any other secondary manifestation connected with *Borrelia* infection, were selected. The goal of the study was the evaluation of the dissemination of *Borrelia* at an early stage of LB infection without clinical systemic involvement. EM is the dermatological hallmark of early LB.

Table II. *Summary of the positive results for each primer set*[1]

Markers	Blood	Urine	Skin (PET)
B-80	47% (14/30)	10% (3/30)	50% (15/30)
66 kDa	53% (16/30)	20% (6/30)	37% (11/30)
Flagellin (41 kDa)	37% (11/30)	13% (4/30)	86% (26/30)

[1]Same patients and samples as in Table I.

EM presents a non-specific histological pattern with dermal lymphocytes infiltration confined mainly to the perivascular area. ELISA is currently the method of choice for laboratory confirmation of LB, but negative serology does not exclude LB, especially in the early phases of the disease. Specific IgM antibodies to *B. burgdorferi* usually appear 3 to 4 weeks after onset of the infection (12), but a prompt antibiotic treatment of the clinical manifestation may sometimes suppress the antibody response. Previous studies have demonstrated that PCR amplification of sequences of the *B. burgdorferi* genome is a valuable tool for supporting the clinical diagnosis of LB (9), especially in the absence of a serologic response in the early stage of the infection (13). PCR can in addition detect non-viable organisms. Thus, a positive PCR result does not establish whether the infection is active or not. In our study, all patients satisfied the Centres for Disease Control and Prevention's Surveillance definition of Lyme disease, and no one was treated with antibiotics in the prior 2 months. Therefore *B. burgdorferi* specific DNA detected in patient's specimens can be considered as confirmation of an active infection.

B. burgdorferi DNA could be detected in 100%

of PET from EM skin by application of the three *Borrelia* genome sequence amplifications. Even if the sensitivity of PCR in PET is lower than in fresh tissues (5), our results demonstrate that with a set of primers it is possible to use PET to detect *Borrelia* DNA reliably. In order to confirm the results in every sample, duplicate tests were performed. Positive results were given only when verified by the second test. In our experience, positivity was confirmed in every case, but sometimes with different intensity.

The rate of positive results is different for distinct primers, especially in PET where the flagellin sequence gave the most frequent positive results. For this sequence, 86% (26 out of 30 cases) were positive for *Borrelia*, which is in agreement with the results obtained by Lebech et al. (14), who detected *Borrelia* DNA in 71% of skin biopsies but only in 13% of urine samples from patients with EM. The reason for the enhanced positivity of this marker in archive tissue is not clear; a possible gene amplification has been suggested. PET-DNA analysis has a lower efficiency than the analysis performed in DNA obtained from fresh tissue, but it is well known that in blood and urine the micro-organism is more diluted (14).

The diagnostic value of urine PCR in early infection is unclear and the previously reported results are controversial (5). It is well known that the diagnostic sensitivity in urine samples is low due to the decreased number of targets (14). The percentages of positive results detected in urine samples using the three different primer sets were comparable and consistent with other reports (14). Also in blood the rate of positive results for the different sequence systems presents no significant differences.

B. burgdorferi spreads locally in the skin, but at the same time probably slips in between the endothelial cells (15, 16). The PCR simultaneously identified *Borrelia* in blood and skin of 23 patients, thereby confirming the suspicion of early dissemination of the pathogen in a major fraction of patients with EM. In a further 9 patients it was detected in both skin and urine. These findings contrast with the usual clinical division of LB into local infection (Stage I that includes EM) and disseminated infection (Stages II and III). According to our results the infections seem to be disseminated already at the initial stages. This spreading of the microorganism at the early phase of the disease concurs with the pathogenesis of syphilis, where *Treponema pallidum* is known to disseminate somatically even at an early stage of the infection (17). Also for this spirochete PCR analysis was reported as a valuable tool for pathogen detection in different types of biological samples (18, 19).

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