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Detection of *Borrelia burgdorferi*-Specific DNA In Urine Specimens from Patients with Erythema Migrans before and after Antibiotic Therapy

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A nested PCR was developed for the detection of *Borrelia burgdorferi*-specific DNA in the urine of patients with erythema migrans. The target for the nested PCR was a specific region of the flagellin gene; the detection limit was less than five organisms of *B. burgdorferi* including all three species *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*. A prospective, controlled, blinded study was performed with 26 patients with erythema migrans to evaluate the nested PCR method with clinical samples. *B. burgdorferi*-specific DNA could be detected in urine specimens from 22 of 24 patients with erythema migrans (sensitivity, 91.67%). Immediately after therapy, 11 of 19 patients still yielded positive results (58%). Eight weeks after therapy, 2 of 16 patients (13%) were positive by PCR of urine, and 20 weeks after treatment none of seven investigated urine samples was reactive. Essential for the sensitivity that was obtained was the development of a simple DNA extraction procedure. The results of the study indicate that the described method is highly sensitive and allows for the effective control of the efficacy of antibiotic therapy in patients with early Lyme borreliosis.

Erythema migrans (EM), a typical skin lesion, is the first clinical sign of infection with *Borrelia burgdorferi* in most patients after a tick bite. This clinical symptom is usually sufficient for diagnosis. However, in a substantial number of patients, this skin eruption may not occur, may be atypical, or may be missed. Under such circumstances, correct laboratory diagnosis becomes important. Current methods, e.g., serology or culture, are unsatisfactory (for a review, see reference 26), and in addition, no single parameter that can be used to monitor the efficacy of antimicrobial therapy is yet available.

PCR has gained importance for the diagnosis of Lyme borreliosis (LB) since the first amplification of a chromosomal DNA of *B. burgdorferi* (20). PCR is able to detect *B. burgdorferi*-specific DNA in a variety of clinical specimens from patients with both early and chronic manifestations of LB. Most reports about the application of PCR to urine specimens from patients with LB refer to patients with neuroborreliosis or late disease (3, 7, 10) and Lyme arthritis (3, 12, 30). PCR has already been performed on serum (5, 12) and skin lesions from patients with EM (11, 16–18, 23, 27, 29), but only a few, preliminary observations exist about investigations of urine (11, 25). We report here on 26 consecutively diagnosed cases of EM in patients residing in an area where borrelial infections are endemic (Styria, Austria). Urine specimens from most of these patients were obtained before and directly after antibiotic treatment. For these patients, additional samples were also acquired 8 and 20 weeks after therapy. Urine specimens were analyzed for *B. burgdorferi*-specific DNA by a newly developed nested PCR procedure.

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MATERIALS AND METHODS

Patients. Twenty-six consecutively diagnosed patients (8 males and 18 females, mean age, 56 years) with EM were seen from April to October 1993 at the University Hospital Department of Dermatology in Graz, Austria. EM was diagnosed on clinical grounds. A 4-mm punch biopsy specimen from the border of the EM lesion was obtained, and specimens from 24 of 26 patients were examined histopathologically to confirm the clinical diagnosis. Patients 22 and 25, who had clinically unequivocal EM lesions, refused a punch biopsy. None of the 26 patients included in the study had received antibiotic therapy. Sixteen patients presented with the annular type of EM (i.e., a peripheral ringlike erythema), and 10 patients presented with the macular type of EM (i.e., a homogeneous erythema with a bluish red center). The mean duration of EM before the first presentation was 27 days (range, 4 to 100 days). Fourteen of 26 patients (54%) were classified as suffering from early disseminated LB and exhibited no additional signs and symptoms. All patients resided in a geographic area where LB is endemic (Styria, Austria). Twenty of 26 patients (77%) could recall an arthropod bite at the same site where EM later developed. The mean time period from the anthropod bite to the appearance of EM was 22 days (range, 4 to 90 days) in these 20 patients. Three patients (patients 2, 4, and 20) had suffered multiple tick or insect bites within an 8-week period before EM occurred (see Table 1). On the first hospital visit, a midstream urine specimen was obtained from all patients.

The patients were reexamined at the end of antibiotic therapy (minocycline at 100 mg orally twice daily for 14 days). Midstream urine specimens could be obtained from 19 patients. A second clinical reevaluation of 22 patients took place 8 weeks after the end of therapy; midstream urine specimens were obtained from 16 of these 22 patients.

Thirteen patients were again reexamined 20 weeks after the end of therapy. Midstream urine specimens were attained from seven of these patients.

Midstream urine specimens from 62 patients with various dermatological disorders (Kaposi's sarcoma, urticaria, and verruca) served as controls.

Bacterial strains. The following strains were used for susceptibility testing: *B. burgdorferi* sensu stricto strains B31 (ATCC 35211), HB1, and H3; *Borrelia garinii* PBi; *Borrelia afzelii* H1, H6, H10, and PKo (PBi and Pko strains were kindly supplied by V. Preac-Mursic, University of Munich, Munich, Germany); *Borrelia japonica* HO14 (kindly supplied by T. Masuzawa, University of Shizuoka, Japan); and five yet untyped strains isolated in Vienna from skin lesions from patients with EM (strains H17, H19, H20, H21, and H22). *Treponema pallidum* Nichols, *Treponema denticola* ATCC 33520, and *Borrelia hermsii* ATCC 35209, in addition to clinical isolates of *Chlamydia trachomatis*, *Gardnerella vaginalis*, *Neisseria gonorrhoeae*, *Mycoplasma hominis*, *Pseudomonas aeruginosa*, and *Ureaplasma urealyticum*, were used to assess the specificity of the nested PCR.

Preparation of urine samples for PCR. Urine specimens were frozen at −70°C, coded, and sent to the laboratory packed in dry ice. After thawing and equilibration at 30°C, 8 ml of the urine samples was centrifuged at 14,000 × *g* for 30 min. The pellet was resuspended in 600 μl of phosphate-buffered saline (PBS;

pH 8.0), and the mixture was stored at −70°C. For analysis, 300 μl was centrifuged at 14,000 × *g* for 20 min and the resulting pellet was dissolved in 50 μl of PBS. A total of 50 μl of a 10% Chelex-100 suspension (Bio-Rad, Richmond, Calif.) was added to the sample before it was heated at 100°C for 5 min and subsequently chilled on ice; after centrifugation (3,000 × *g*, 1 min), 10 μl of the supernatant was used as the source of template DNA in the first PCR.

Nested PCR assay. Two nested PCRs, a heminested PCR and a nested PCR targeting the chromosomal flagellin gene of *B. burgdorferi* (GenBank accession no. X15661 [2]), were tested. In the heminested PCR assay (PCR-A in Fig. 1), an outer primer pair, BBSCH1 (AGC ATC ACT TTC AGG GTC TC) and BBSCH2 (TGT CAT TGT AGC ATC TTT TAT TT), representing positions 483 to 502 and 903 to 881, respectively, and an inner primer pair, BBSCH1 and BBSCH4 (CCT CAC CAG AGA AAA GAT T), representing positions 602 to 584, were used. The first PCR with the outer primers was run for 25 cycles with an annealing temperature of 52°C (30 s) in a total volume of 60 μl. Elongation and denaturation took place at 72°C for 60 s and at 94°C for 30 s. The reaction mixture contained 50 mM KCl, 1.5 mM magnesium chloride, 10 mM Tris-HCl (pH 8.3), 0.05% glycerol, 0.1% Triton X-100, 0.1 mM EDTA, 0.1% bovine serum albumin, 200 μM (each) desoxynucleotide triphosphate (dAMP, dCTP, dTTP, and dGTP), 50 pM (each) outer primers, 2.0 U of *Taq* polymerase (Amplitaq; Perkin-Elmer, Norwalk, Conn.), and 10 μl of the DNA preparation. The mixture was overlaid with 1 drop of mineral oil. The amplification reactions were performed in 200-μl thin-walled tubes with attached caps either in a Gene AMP PCR system 9600 (Perkin-Elmer) or in a PTC-100-96 thermocycler with a hot bonnet (MJ-Research; Watertown, Mass.). Three microliters from the first PCR mixture was transferred with a pipette with a disposable piston into the 50-μl reaction mixture of the second PCR mixture containing the same reactants used in the first PCR mixture, but with 25 pM (each) the inner primers. The second PCR was done with 35 cycles of the same durations and at the same temperatures as those used in the first PCR, yielding 120-bp product.

For the nested PCR (PCR-B in Fig. 1), 50 pM (each) the outer primers, of the flagellin gene (2), primers BBSCH31 (CAC ACC AGC ATC ACT TTC AGG GTC T) and BBSCH42 (CAA CCT CAT CTG TCA TTG TAG CAT CTT TTA TTT), representing positions 477 to 501 and 913 to 881, respectively, the same master mixture used in the first PCR, and 10 μl of DNA preparation were used. The total volume was adjusted to 60 μl with water. The mixture was overlaid with 1 drop of mineral oil. The amplification reactions of the first PCR consisted of 25 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Thermocycling was preceded by 1 min at 95°C; this was followed by a final 10 min of extension at 72°C. After amplification, 3 μl of the first PCR mixture was transferred to a second PCR mixture consisting of the same master mixture described above, but instead of outer primers, 25 pM (each) the inner primers FL-59 (TTT CAG GGT CTC AGG CGT CTT) and FL-7 (GCA TTT TCA ATT TTA GCA AGT GAT G), representing positions 491 to 511 and 767 to 743, respectively, as modified from the primers of Picken (19), were used, yielding a 277-bp amplicon. The second PCR was done with 35 cycles of the same durations and at the same temperatures used for the first PCR for denaturation and elongation, but the annealing temperature was increased to 58°C.

The same number of blank controls, which contained 5 μl of water substituted for DNA, as patient samples, as well as a positive control sample with 50 copies of *B. burgdorferi* B31 DNA and DNA samples from uninfected patients as well as two infected patients, were run in parallel with each amplification assay. If any of the blank controls or samples from uninfected patients was found to be positive, the whole run was repeated. Positive results were verified by a repeat PCR starting with a second urine extraction. Negative results were checked for inhibition by a newly developed assay.

Extraction of DNA, preparation of PCR mixtures, amplification, and analysis of the amplicons were each done in separate areas. Filter-barrier pipette tips and a dedicated set of pipettors were used to prepare all samples. Setting up of the inner PCR was done in a PCR workstation (CBS, Del Mar, Calif.), which was used only for this reaction step. Positive-displacement pipettes with disposable pistons were used to prepare the PCR mixture. After each run the workstation was cleaned with an aqueous hypochloric solution.

Amplified products (10 μl of hypochlorite) were resolved by 2% agarose gel electrophoresis (NuSieve/SeaKem, 3:1; FMC Corporation, Rockland, Maine) at 150 V for 25 min. The gel was then stained with ethidium bromide and the bands were visualized under UV illumination at 254 nm.

All laboratory investigations were performed without knowledge of donor clinical data, nor was it known if the urine was from a presumably infected patient or from a control subject.

Inhibition assay. In order to exclude inhibition of the polymerase reaction for samples with nonreactive results, all PCR-negative samples were analyzed as follows. A total of 100 copies of an internal standard were added to the first PCR mixture. This standard was constructed by designing 40-mer primers for a 240-bp nonhomologous region of a 0.6 kb *Bam*HI-*Eco*RI fragment of the v-*erbB* gene (PCR MIMIC Construction Kit; catalog no. K1700-1; Clontech, Palo Alto, Calif.) (24). The inner 20 oligonucleotides were complementary to the v-*erbB* fragment, and the outer 20 oligonucleotides were complementary to the *B. burgdorferi* amplicon generated in the first PCR. With this internal standard, specific primers BBSCH1-BBSCH2 amplify a 240-bp amplicon, if no inhibition of the polymerase occurs. All samples that failed to amplify a 240-bp product were considered inhibitory and were excluded from the study.

Serology. Analysis for immunoglobulin G (IgG) and IgM antibodies to *B. burgdorferi* was performed on the day of the first hospital visit and routinely at the first and third reexaminations. Purified, native flagellum of *B. burgdorferi* DK-1 isolated from a human EM lesion (DAKOPATTS ELISA Kit; DAKO Diagnostika GmbH, Hamburg, Germany) was used as the test antigen (6).

RESULTS

All *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica* isolates and the five yet untyped strains isolated from skin lesions from patients with EM in Vienna could be amplified by both nested PCRs. The detection limit was less than five organisms per PCR for all strains tested. Under the experimental conditions, no reaction occurred with *T. pallidum*, *T. denticola*, *B. hermsii*, *C. trachomatis*, *G. vaginalis*, *N. gonorrhoeae*, *M. hominis*, *P. aeruginosa*, or *U. urealyticum* (data not shown).

B. burgdorferi-specific DNA was found in 22 of 24 urine samples from untreated patients with EM by both nested PCRs (Table 1). However, because of the higher annealing temperature of PCR-B, the resulting 277-bp amplicon was always strong, whereas the 120-bp amplicon of the heminested PCR-A was generally weaker and the result of PCR-A was classified as borderline for three patients (patients 4, 5, and 14 in Fig. 1). Essential for the high sensitivity of the nested PCR was the DNA preparation, because only samples boiled in the presence of Chelex-100 showed specific amplicons (Fig. 1).

Inhibition was found for 2 of the 26 urine samples tested completely (from patients 17 and 21), and the results for these samples had to be excluded from the analysis. Three of the 62 control urine samples were positive, and additional urine samples taken from these patients, one with a diagnosis of Kaposi's sarcoma, one with scleroderma, and one with papules on the nose, all of which were antibody negative and without a history of tick bite, were found to be nonreactive, indicating carryover contamination from the first urine samples. All three false-positive urine samples were found within the first 2 weeks of the study, and precautions for avoiding contaminations were, up to then, found to be effective, because no other single false-positive samples could be detected during the study. Thus, the following values concerning the nested PCR applied in the present study could be calculated: sensitivity, 91.67%; specificity, 95.16%.

Thirteen of the 22 patients (59%) with a reactive urine sample showed additional signs and symptoms as a result of early disseminated LB. Nine of the 22 patients (41%) had no extracutaneous manifestations. One patient (patient 25), for whom the urine PCR yielded a negative result, presented with flulike symptoms. The second patient (patient 13) with a negative urine PCR result was otherwise healthy. No differences regarding further anamnestic or clinical data between patients with reactive and nonreactive urine samples could be found.

IgG antibodies to *B. burgdorferi* were found by enzyme-linked immunosorbent assay (ELISA) in the sera of 8 of the 22 patients (36%) whose urine tested positive by PCR; IgM antibodies in the sera of 3 of these patients (14%) were found by ELISA. On the other hand, a positive IgG antibody titer for *B. burgdorferi* could be detected in one of the two patients with a negative urine PCR result, both of whom had a negative result for *B. burgdorferi* IgM antibodies.

The results obtained after treatment are summarized in Table 2. Eleven of 19 urine samples obtained directly after antibiotic therapy contained *B. burgdorferi*-specific DNA (58%). The PCR result for all of these 11 patients before therapy was also positive. The urine of six patients that initially yielded a positive PCR result showed no more reactivity directly after therapy. For nine patients no direct comparison was possible because of missing data or inhibition, as mentioned above. Three of 11 patients (27%) with PCR-reactive urine samples directly after therapy had a residual erythema and slight extra-

TABLE 1. Clinical and laboratory data for patients with EM before antibiotic therapy[a]

Patient no.	Sex	Age (yr)	Arthropod bite	Time from arthropod bite to EM (days)	Site of EM	Type of EM	Size of EM (cm)	Duration of EM before first visit (days)	Additional signs and symptoms	S *Bb* IgG Ab	S *Bb* IgM Ab	*Bb* DNA in urine specimen
1	F	48	T	28	Right shoulder	M	16 × 16	28	N	+	+	+
2	F	63	T[b]	UK	Right buttock	A	21 × 12	60	H, M	+	+	+
3	F	70	T	60	Lower abdomen	A	60 × 16	15	N	−	−	+
4	F	79	I[b]	UK	Left thigh	A	12 × 7	6	F	−	−	+
5	F	61	T	90	Lower abdomen	M	20 × 15	40	N	+	−	+
6	F	53	T	11	Right thigh	A	15 × 11	10	ST	−	−	+
7	F	81	T	8	Left thigh	M	26 × 26	20	N	+	−	+
8	F	59	T	8	Right knee	M	10 × 6	4	N	−	−	+
9	F	49	I	14	Right shoulder	A	24 × 16	28	H, AR	−	−	+
10	F	60	I	12	Lower abdomen	A	22 × 12	18	J, M	−	−	+
11	F	84			Left shoulder	A	30 × 20	25	N	−	−	+
12	F	54	T	14	Left thigh	A	29 × 29	70	L	+	−	+
13	M	57			Upper thorax	A	16 × 14	21	N	−	−	−
14	F	34	I	5	Right thigh	A	13 × 10	6	N	−	−	+
15	F	29	T	30	Lower back	A	34 × 28	28	F, AR	+	+	+
16	M	46	I	10	Right thigh	M	16 × 16	7	F, H	+	−	+
17	F	52	T	25	Right buttock	A	17 × 15	42	N	−	−	Inhibited
18	F	56	I	7	Right thigh	A	17 × 11	21	AR	−	−	+
19	M	55			Right thigh	A	16 × 15	25	TE, F, AR, M	−	−	+
20	M	51	T[b]	UK	Left thigh	M	16 × 6	22	F, H	−	−	+
21	M	52	T	30	Left upper arm	A	14 × 11	100	N	+	−	Inhibited
22	M	70	T	5	Left knee	M	30 × 17	10	N	+	−	+
23	M	63	T	40	Upper thorax	A	20 × 12	26	N	−	−	+
24	M	71	I	4	Right buttock	M	7 × 6	17	TE	−	−	+
25	F	20	T	10	Lower abdomen	M	7 × 5	4	TE, F, SR, H, M	+	−	−
26	F	36	T	26	Lower back	M	18 × 8	50	F, H	−	−	+

[a] Abbreviations: F, female; M, male, T, tick; I, insect; UK, unknown, M, macular type of EM; A, annular type of EM; N, none; H, headache; M, myalgias; F, fatigue; ST, sore throat; AR, arthralgias; J, temporomandibular pain; L, regional lymphadenopathy, TE, fever; *Bb*, *B. burgdorferi*; Ab, antibodies. A total of 26 patients were examined.
[b] Multiple tick or insect bites. Date of last one could not be recalled exactly.

cutaneous symptoms. Five of these 11 patients (46%) showed a residual erythema but suffered from no additional signs and symptoms. Another three patients were completely healthy and their EM lesions had totally cleared. Three of the six patients who had a negative urine PCR result directly after therapy still had a residual erythema, and one of them had a residual erythema plus headache; the other two patients had no clinical symptoms.

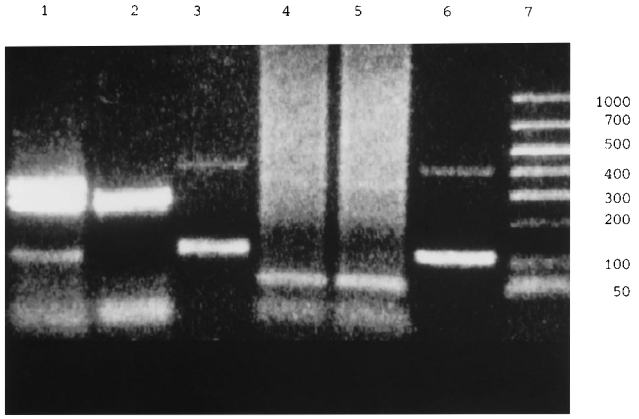

FIG. 1. Influence of DNA preparation on PCR. A centrifuged and washed urine sample from one infected patient was boiled with (lanes 2 and 3) or without (lanes 4 and 5) Chelex-100 and was amplified by heminested PCR (PCR-A; lanes 3 and 5) and nested PCR (PCR-B; lanes 2 and 4). Electrophoretic separation of PCR products on a 2% agarose gel is shown together with two markers, a homemade marker with 120-, 277-, and 341-bp fragments (lane 1) and a marker from Bio Ventures, Inc., Murfreesboro, Tenn., with linear, double-stranded DNA bands from 50 to 1,000 bp, as indicated on the right (lane 7). Lane 6, positive control. *B. burgdorferi*-specific bands (120 bp by heminested PCR and 277 bp by nested PCR) can be seen only with the DNA preparation that included Chelex-100.

A positive IgG antibody titer for *B. burgdorferi* was measured by ELISA in the sera of 4 of the 11 urine PCR-positive patients (36%). On the other hand, a positive IgG antibody titer for *B. burgdorferi* could be detected by ELISA in the sera of three of the eight patients (38%) with a negative urine PCR result. A positive IgM titer could be found in the serum of only one patient (patient 8), who was clinically healthy and who still had a positive urine PCR result.

Twenty-two patients were again seen 8 weeks after therapy. For 16 of these 22 patients, midstream urine specimens were investigated for *B. burgdorferi*-specific DNA by PCR. Specimens from only 2 of these 16 patients (13%) were still positive. However, both patient with positive urine specimens (patients 15 and 16) were completely healthy at this time and no longer had EM lesions. Serological data were not available for these patients. On the other hand, patient 6, who still had residual EM lesions 8 weeks after treatment, did not show any *B. burgdorferi*-specific DNA in his urine sample.

Urine samples for PCR investigation could be obtained from 7 of 13 patients who could be followed up until 20 weeks after the end of antibiotic treatment. These specimens, all of which were obtained from then clinically healthy patients, yielded negative results. Interestingly, the sera of two of the patients (patients 3 and 6) had a positive IgM antibody titer for *B. burgdorferi* by ELISA, in contrast to their preceding serologic analyses. The serum of another urine PCR-negative patient (patient 16) still had a positive IgG antibody titer for *B. burgdorferi* ELISA.

DISCUSSION

PCR-based technology has been successfully used for the detection of *B. burgdorferi* specific DNA in patients with dif-

TABLE 2. Clinical and laboratory data for patients with EM after antibiotic therapy[a]

Patient no.	Directly after therapy					8 wk after therapy					20 wk after therapy				
	Appearance of EM	Additional signs and symptoms	S *Bb* IgG Ab	S *Bb* IgM Ab	*Bb* DNA in urine specimen	Appearance of EM	Additional signs and symptoms	S *Bb* IgG Ab	S *Bb* IgM Ab	*Bb* DNA in urine specimen	Appearance of EM	Additional signs and symptoms	S *Bb* IgG Ab	S *Bb* IgM Ab	*Bb* DNA in urine specimen
1	RE	N	+	−	−	C	N	ND	ND	−	C	N	+	−	ND
2	RE	N	ND	ND	ND	C	N	ND	ND	ND	C	N	ND	ND	ND
3	RE	N	−	−	ND	C	N	ND	ND	−	C	N	−	+	−
4	RE	N	−	−	+	—	—	—	—	—	—	—	—	—	—
5	RE	N	−	−	ND	RE	N	ND	ND	ND	C	N	+	−	ND
6	RE	N	−	−	−	RE	N	ND	ND	−	C	N	−	+	−
7	RE	N	+	−	+	C	N	+	−	ND	—	—	—	—	—
8	C	N	−	+	+	C	N	−	−	ND	C	N	−	−	ND
9	C	N	−	−	−	C	N	ND	ND	−	C	N	−	−	−
10	RE	N	−	−	+	C	N	ND	ND	−	C	N	−	−	ND
11	C	N	+	−	+	—	—	—	—	—	—	—	—	—	—
12	C	N	+	−	−	C	N	ND	ND	ND	—	—	—	—	—
13	C	N	ND	ND	ND	C	N	+	−	−	—	—	—	—	—
14	RE	N	−	−	+	C	N	ND	ND	−	—	—	—	—	—
15	C	N	+	−	+	C	N	ND	ND	+	—	—	—	—	—
16	RE	H	+	−	+	C	N	ND	ND	+	C	N	+	−	−
17	C	N	−	−	−	C	N	ND	ND	−	—	—	—	—	—
18	RE	A	−	−	+	C	N	ND	ND	−	—	—	—	—	—
19	RE	H	−	−	−	C	N	−	−	−	C	N	−	−	ND
20	RE	H	−	−	+	C	N	ND	ND	−	C	N	−	−	−
21	C	N	+	−	−	C	N	ND	ND	−	C	N	−	−	−
22	RE	N	+	−	ND	—	—	—	—	—	—	—	—	—	—
23	RE	N	−	−	+	C	N	ND	ND	−	—	—	—	—	—
24	RE	N	−	−	−	C	N	−	ND	ND	C	N	−	−	−
25	RE	H	ND	ND	ND	—	—	—	—	—	—	—	—	—	—
26	RE	F	−	−	ND	C	N	ND	ND	−	—	—	—	—	—

[a] Abbreviations: RE, residual erythema (faded, diminished); C, EM completely cleared; N, none; H, headache; A, arthralgias; F, fatigue; S, serum; *Bb*, *B. burgdorferi*; Ab, antibodies; ND, not done; —, loss from follow-up. A total of 26 patients were examined.

ferent manifestations of LB in a variety of body tissues and fluids. There are reports of PCR investigations of serum (5, 12), blood (25), and skin lesions (11, 16, 23, 25, 27, 29) from patients with EM. *B. burgdorferi* disseminates early in the course of the disease. Thus, urine from patients with EM also might be a suitable clinical source for *B. burgdorferi*-specific PCR. The following facts support this assumption. (i) *B. burgdorferi* DNA could be found by culture and PCR in renal and urinary bladder tissue from white-footed mice (*Peromyscus leucopus*) living in areas where LB is endemic (14, 22). (ii) The detection of *B. burgdorferi* components in urine by immune capture methods or inhibition ELISA has been reported for animal models and patients with LB (1, 8). Magnarelli and coworkers (13) recently found *B. burgdorferi* antigens in urine specimens from 8 of 28 patients with EM by an inhibition ELISA (28.6%). (iii) Very low numbers of *B. burgdorferi* organisms could be detected in urine samples in vitro by PCR analysis (15). Furthermore, multiple urine specimens can easily be obtained during the course of the disease.

However, only a few, preliminary reports exist about the application of PCR to urine specimens in humans with early LB. Lebech et al. (11) found *B. burgdorferi*-specific DNA in urine samples from 3 of 29 (10%) untreated, consecutively diagnosed patients with EM by using a PCR based on the amplification of a fragment of the 16S rRNA gene. Sun et al. (25) obtained positive results for 18 and 21% of 23 patients with EM by testing pretreatment urine samples in duplicate by the same method used by Lebech et al. (11). The authors concluded from this low sensitivity that urine is not a suitable reservoir for the detection of *B. burgdorferi*.

In order to improve *B. burgdorferi* detection by PCR, two assumptions had to be proved: (i) All three species of *B. burgdorferi*, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, should be detected with similar sensitivities. This was possible with targets from the flagellin gene. Recently, it was shown (17) that the sensitivity of PCR with the *ospA* gene as the target varied by up to 10^3 with different strains. (ii) Sensitivity should be high in order to obtain reproducible results for specimens containing only a limited number of organisms.

Urine samples from infected patients amplified by standard PCR gave inconsistent results, and sensitivity was low (<40%; data not shown). Therefore, nested PCRs were tested. In addition to their higher levels of sensitivity and specificity, nested PCRs have been shown to be able to replace hybridization reactions for confirmation of the results (31). The heminested PCR-A was highly sensitive and specific; however, with clinical samples, the 120-bp agarose band was sometimes very weak. The improved nested PCR-B was designed with OLIGO software (21). Outer primers BBSCH31-BBSCH42 were modified from the outer primers of the nested PCR-A, and inner primers were modified from the work of Picken (19). Both annealing temperatures of nested PCR-B could be increased with this modification.

Carryover contamination may be a problem, especially in nested PCR. In order to follow known recommendations for avoiding contamination (9), the following steps were essential: (i) parallel testing of high numbers of blank controls, (ii) use of positive controls with low copy numbers (50 to 100 copies), (iii) use of oil overlays in the first and second PCRs (iv) use of only reaction tubes with attached caps, not microtiter plates sealed with either adhesives or thermoplastic cover sheets and no cap strips, and (v) use of a DNA extraction procedure with as few handling steps as possible. In the present study such precautions were apparently efficient, since urine from only 3 of 62 control subjects was reactive.

Urine is known to contain inhibitors of *Taq* polymerase (4). Recently, inhibition was seen in 7 of 10 urine samples (7). We have seen inhibition only for urine from 2 of 26 patients. The advantage may be due to the incorporation of the Chelex-100 resin (28) in the DNA preparation and to the nested PCR: by transferring only 1/20 of the reaction mixture of the first PCR mixture into the second PCR mixture, possible inhibitors were also diluted out to the same extent.

Our urine PCR was positive for 22 of 24 untreated patients with EM (sensitivity, 91.67%). Comparison of the reactivity of the urine PCR with that of serological testing in our patients with EM reveals a substantial lack of correlation. Of the 22 patients with a positive urine PCR result, sera from only 8 patients (36%) showed increased IgG antibody titers to *B. burgdorferi* by ELISA. Three of these eight patients (14%) also had IgM antibodies. Thus, *B. burgdorferi* in 14 serology negative patients could be diagnosed only by urine PCR. On the other hand, only one patient (patient 25) showed a positive serology result but a negative PCR result.

Eight weeks after therapy, 2 of 16 patients (13%) had a reactive urine PCR. Interestingly, these two patients were completely healthy, whereas one patient with residual erythema (patient 6) already had a negative urine PCR result. Apart from these three patients, there was complete correlation between the patient's clinical condition and the PCR results. Twenty weeks after treatment, none of seven investigated urine samples from then clinically healthy patients was reactive.

On the basis of the results of our study, the following conclusions can be drawn. (i) *B. burgdorferi*-specific DNA can be detected sensitively in urine specimens of patients with EM by the nested PCR procedure described here. (ii) Urine PCR appears to be a sensitive and specific diagnostic tool for early LB. However, negative results do not rule out actual LB. (iii) Urine PCR for *B. burgdorferi* can be reactive within the first week of the presence of EM. (iv) PCR results become negative weeks after adequate antibiotic treatment. Urine PCR might therefore be a useful method for long-term monitoring of the efficacy of antibiotic therapy for patients with EM, but it cannot substitute for clinical judgment.

Additional studies with larger numbers of patients and investigations at different times during and after therapy are needed to further assess the use of urine PCR as a diagnostic tool for early LB.

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