

CLINICAL REPORT

Course of *Borrelia burgdorferi* DNA Shedding in Urine after Treatment

Elisabeth ABERER¹, Andreas R. BERGMANN¹, Anna-Maria DERLER² and Bruno SCHMIDT³

Departments of Dermatology, ¹Medical University of Graz, ²Medical University of Innsbruck, and ³Lainz Hospital, Vienna, Austria

Diagnosis of Lyme borreliosis by urine polymerase chain reaction (PCR) has been recognized as having better diagnostic sensitivity in patients with erythema migrans than serological methods. We made serial tests with 192 urine specimens from 70 patients with erythema migrans and 60 urine specimens from 21 patients with acrodermatitis chronica atrophicans to evaluate the course of positive urine PCR after antibiotic treatment. Before treatment, urine samples from patients with erythema migrans showed a positive PCR in 27/34 samples (79%), and those from patients with acrodermatitis chronica atrophicans in 7/11 (63%). The specificity of bands was proven by hybridization with GEN-ETI-K™-DEIA kit in 40/41 samples. Borrelia DNA in urine decreased gradually within the observation period of one year in both patients with erythema migrans and acrodermatitis chronica atrophicans, and persisted without clinical symptoms in 4/45 patients with erythema migrans (8%) after 12 months. Urine PCR can serve as a diagnostic method in early Lyme borreliosis and also in seropositive patients with unclear clinical symptoms. Key words: acrodermatitis chronica atrophicans; erythema migrans; Lyme borreliosis; PCR.

(Accepted August 18, 2006.)

Acta Derm Venereol 2007; 87: 39–42.

Elisabeth Aberer, MD, Department of Dermatology, Medical University of Graz, Auenbrugger Platz 8, AT-8036 Graz, Austria. E-mail: elisabeth.aberer@meduni-graz.at

Lyme borreliosis (LB) is generally diagnosed by serological methods, which are easy to use for routine testing (1). In early LB, however, antibodies can only be detected in 40–60% of patients (2, 3). In contrast, high seropositivity in healthy individuals (4) and persistence of antibodies after LB has been treated make it difficult to distinguish between active, persisting and previous infection. The most sensitive method for confirming a borrelia infection has been found to be quantitative polymerase chain reaction (PCR) on skin biopsies (80.9%), followed by two-stage serological testing of convalescent-phase samples (66.0%), nested PCR on skin biopsies (63.8%) and culture (51.1%) (3). In various studies, PCR for *B. burgdorferi* has been performed on organs, cultures,

culture supernatants, and body fluids in clinical studies, animal models, and ticks using different targets for amplification derived from chromosomal- and plasmid-related molecules (5). Whereas amplification of borrelia DNA from synovial and cerebrospinal fluid has already become an important diagnostic tool (6–10), findings of a positive urine PCR have also been reported repeatedly in recent years (11–17). In previous investigations, the PCR technique to detect *B. burgdorferi* DNA in urine samples was revised. Conditions for handling, storage and extraction have been found to be crucial for PCR methods using patient material (12). It is essential to avoid the first morning urine, to centrifuge at 36.000×g, to extract with DNAzol, and not to freeze for longer than 3 months. When handled accordingly, urine samples from untreated patients with erythema migrans (EM) were positive in 85%.

The aim of this study was to follow patients with EM and with acrodermatitis chronica atrophicans (ACA) for one year and to compare clinical data with the results of urine PCR.

MATERIALS AND METHODS

Patients

Serial examinations avoiding morning urine were performed with 192 specimens from 70 patients (40 females and 30 males, 49.3 years (range 9–81)) with EM, 60 specimens from 21 patients with ACA, and 59 specimens from healthy control individuals. This study was approved by the local ethics committee. EM was diagnosed clinically using the EUCALB criteria (2). Nineteen of 70 patients with EM presented with disseminated disease showing multiple EMs, headache, arthralgia and/or fatigue. The incubation time was known in 64/70 patients and averaged 60 days (median 48, range 7–365 days) upon first presentation. All patients with ACA (16 females and 5 males, 62 years (range 31–97)) were seropositive and the diagnosis was confirmed by histology according to the EUCALB criteria. Patients with EM were treated with penicillin or tetracycline for 2 or 3 weeks, patients with ACA for 30 days with the same antibiotics.

Urine specimens of EM patients were examined before treatment ($n = 34$), then 5–30 days ($n = 47$), 1–3 months ($n = 28$), 4–6 months ($n = 20$), 7–9 months ($n = 18$), and 10–12 months ($n = 45$) after treatment. In ACA patients, the PCR positive urine samples were re-tested at the same time period. Individuals' urine samples were tested between one and six times, for a mean of 2.9 times. Antibodies were investigated by IDEIA flagellum *B. burgdorferi* ELISA (Dakopatts, Copenhagen, Denmark). IgM and/or IgG titres were calculated to evaluate seropositivity.

Methods

Ten ml aliquots of urine were frozen at -80°C until use, thawed at room temperature or subjected immediately to extraction. Ten ml of urine were centrifuged at $36.000\times g$ at 4°C for 30 min. Genomic DNA was isolated from the pellet with 1 ml of a guanidine-detergent lysing solution (DNAzol[®], Molecular Research Center, Inc., Cincinnati, Ohio) that hydrolyses RNA and allows the selective precipitation of DNA. The solution was then mixed with 6 μl of a polyacryl carrier (microcarrier[™] Gel-TR, MRC). After 10 min, DNA was precipitated from the lysate with 500 μl ethanol p.a. and centrifuged as described above for 20 min. The pellet was washed twice with 1 ml 75% ethanol, the supernatant discarded in each case, and the DNA then solubilized in 100 μl ultrapure water.

For nested PCR, 10 μl of the sample were subjected to the first PCR. Amplitaq Gold Polymerase (1.25 U / reaction; Perkin-Elmer-Cetus, CT, USA) and Gold buffer (Applied Biosystems, Foster City, CA, USA) were used. Nested PCR was performed with a set of one outer and one inner primer pair (BBSCH1/BBSCH2 and FL7/FL59) targeting portions of the inner part of the *B. burgdorferi* flagellin gene (11). The flagellin PCR cyclor program consisted of 30 cycles with denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec. Thermocycling was preceded by a 10-min phase at 95°C . The final extension phase lasted for 10 min at 72°C . Four μl from the obtained amplicons were used for the second PCR with the same cyclor program but an annealing temperature of 58°C . To avoid cross-contamination and sample carryover, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms, and plugged pipette tips were used for all fluid transfers. Amplicons were visualized at 276 bp on 3% agarose gels stained with ethidium bromide.

For hybridization, positive amplicons were verified in 41 samples, 38 from patients with EM and 3 from patients with ACA by GEN-ETI-K[™]-DEIA (Diasorin Inc., Stillwater, MN, USA) applied according to the manufacturer's protocol. This DNA enzyme immunoassay is based on the hybridization of amplified DNA with an oligonucleotide probe (F1 15 and F1 16, corresponding to *B. afzelii* or *B. garinii* genospecies), complementary to a sequence of the amplified DNA that is coated on the wells of a microtitre plate by a streptavidin-biotin bridge (cross-reactions between these genospecies cannot be avoided by this assay because of the highly conserved flagellin gene sequence). Single-stranded sample DNA (heat denatured) is added to the well and hybrids formed are detected by an anti-DNA mouse monoclonal antibody that reacts only with double-stranded and not with single-stranded DNA; the absorbance (OD) is then measured with a spectrophotometer at 450/620 nm.

RESULTS

In this study, urine PCR was positive in 27/34 (78%) tested EM patients before treatment. There was a gradual decrease to 65% after treatment, to 32% after 3 months, 20% after 6 months, 16% after 9 months, and to 8% after one year. This latter percentage is higher than in healthy individuals, of whom 2/59 (3%) were positive. In Fig. 1 the course of *B. burgdorferi* DNA-shedding in urine is illustrated. The difference in DNA-shedding depending on disseminated disease (Fig. 1a), the duration of treatment (Fig. 1c) and the given antibiotic (Fig. 1b) is presented. In one patient, persisting positive PCR was detected in 5 serial tests up to 18 months but

urine was negative after 3 further months (21 months in all). Another patient was positive 12 months after treatment, but negative after 16 months. A further patient was positive before and after treatment and after 12 months, but negative after 3 and 6, and then again after 21 months. A fourth positive patient was only tested twice, before treatment and after 12 months.

As to the 4 patients with persisting positive urine PCR, 3 of them had disseminated EM, and/or were among the 2-week and penicillin-treated patients (Fig. 1b, 1c). All these patients, however, were free of clinical symptoms in the follow-up period.

An apparent increase in positive urine PCR after 9 months as depicted in Fig. 1c is possibly due to low sample numbers because DNA was re-detected in urine after 6 and 9 months in only one patient. Paired urine and serological tests revealed that before treatment, 18/22 seronegative and 9/12 seropositive patients were PCR positive (Fig. 2). Altogether antibodies were detected in 12/34 patients (35%) before treatment and in 12/44 (27%) after one year, whereas the urine PCR showed a gradual decline in *B. burgdorferi* DNA from 79% before treatment to 8% (in 4/45 patients) after one year. At that time, these 4 patients were seronegative. In the urine samples of ACA patients, positive before treatment in 7/11 (63%) samples a gradual decrease in DNA shedding was observed during one year (Fig. 1d). Their clinical course was unremarkable, with a slow decrease of livid discoloration during the months after treatment.

Hybridization with GEN-ETI-K[™]-DEIA allowed identification of *B. afzelii* and/or *B. garinii* in 40/41 samples. Because of the highly conserved flagellin gene sequence, there can be cross-reactions between these oligonucleotides (12).

DISCUSSION

This study detected a decline in DNA-shedding in urine from patients with EM and ACA during a 1-year observation period. In contrast to our previous investigations with 85% positive samples (12), only 79% of urine specimens from the patients with EM in the present study were positive before treatment. This might be due to problems with storing and inhibition, which have been found to be crucial for a successful DNA assay. Urine samples were not investigated immediately and freezing specimens for more than 3 months reduced sensitivity to 24.8% (12).

In a recent prospective study by Mercier et al. (17), urine PCR was found to be positive in 12/12 patients with EM. Five weeks after therapy, all urine samples were negative. In another study, urine samples were also negative after treatment (18). Since repeated (up to 6) urine tests were positive in our patients, we find it plausible that there is a gradual decrease in DNA fragments in the organism after treatment. Furthermore,

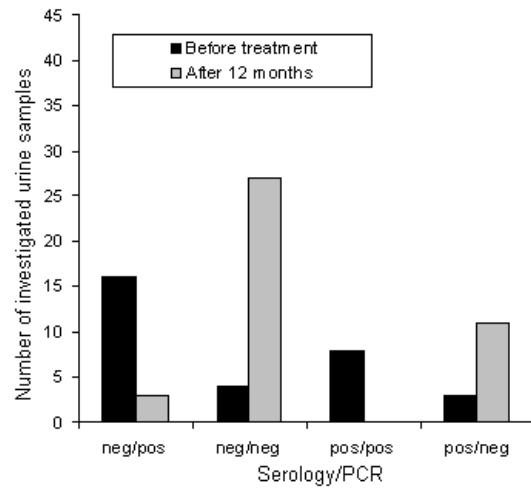
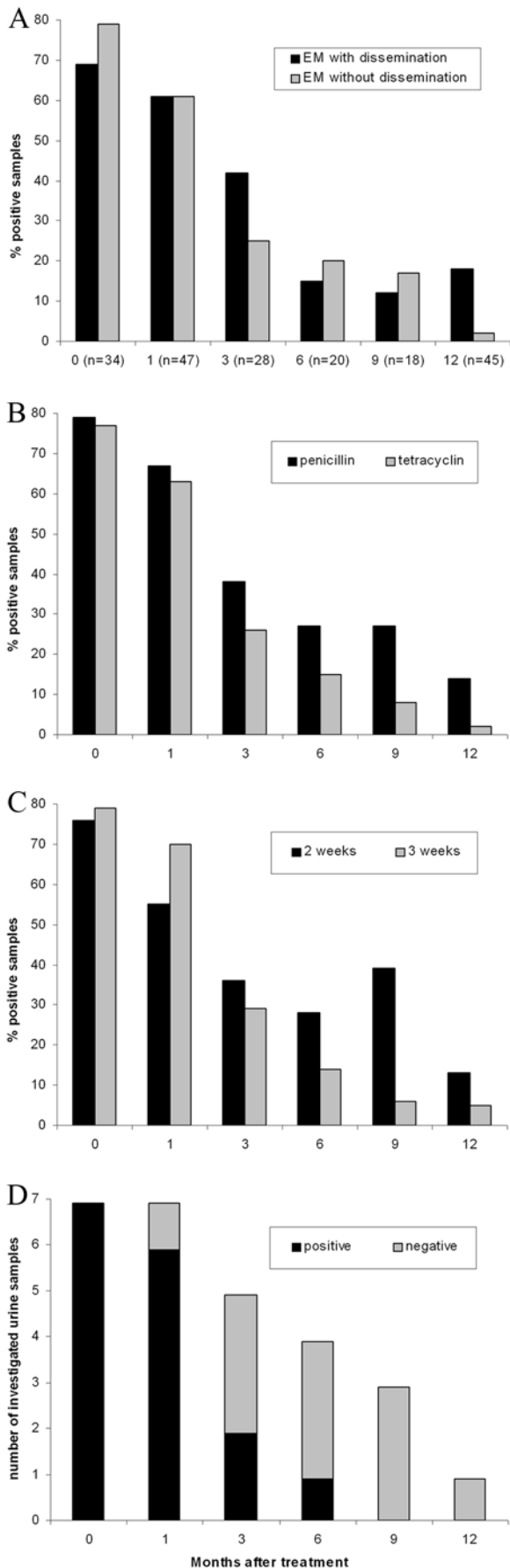


Fig. 2. Comparison of serology and PCR results before treatment and after 12 months in patients with erythema migrans.

since PCR can also detect DNA from dead organisms, the infection itself could have long since cleared up. Slow elimination of DNA has also been reported in syphilis, but a survival of treponema after treatment is well known (19, 20). In 3/4 patients, it took 16–21 months for urine PCR to become negative. The rate of positive healthy individuals was 3%. In these persons interviewed by a questionnaire, LB could be ruled out and this latent *B. burgdorferi* infection is not associated with clinical symptoms. It is well known that EM can show spontaneous regression without subsequent clinical disease, and asymptomatic persistent infection may occur in seropositive persons (21–23).

Apart from the conditions for a successful PCR as elaborated in an earlier study, problems arise due to the low number of DNA copies present in urine. In recent experiments aimed at simplifying the urine PCR technique, we came to realize that borrelia DNA cannot be detected by one-step real-time PCR in patient samples since the requirements for positive patient samples are five borrelia cells/PCR and with real-time PCR, the detection rate was 50 borrelia cells/PCR (24), meaning that nested PCR works at the detection limit.

The urine PCR method is not yet standardized. Heterogeneous techniques, extractions, and primers have been used with variable results (12–18). An important step for verification of detected bands on agar electrophoresis is sequencing or hybridization. In 40/41 positive PCRs, hybridization with GEN-ETI-K™-DEIA showed sequences for *B. afzelii* and/or *B. garinii* that can show cross-reactions (12).

Fig. 1. The course of *Borrelia burgdorferi* DNA shedding in urine after treatment. a) comparison of urine PCR in patients with or without dissemination, b) after penicillin vs. tetracycline treatment, c) after 2 or 3 weeks treatment, d) serial investigations of PCR-positive ACA patients.

Urine PCR is an interesting method, which is advantageous for the diagnosis of borrelia infections. Further standardization is necessary. Molecular diagnosis of a *B. burgdorferi* infection from urine can help to identify seronegative patients with clinical symptoms of LB, and can evaluate seropositive patients for a persistent infection and guide antibiotic treatment. Furthermore, urine investigation is a non-invasive method that could also identify patients with suspected Lyme arthritis or neuroborreliosis.

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