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MOLECULAR AND SEROLOGICAL DIAGNOSIS OF *BORRELIA BURGDORFERI* INFECTION AMONG PATIENTS WITH DIAGNOSED *ERYTHEMA MIGRANS*

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Abstract: The aim of the study was to assess the frequency of *Borrelia burgdorferi* DNA detection in the blood and urine of patients diagnosed with *erythema migrans*, and compare the results of PCR-based methods with ELISA methodology. The latter was used to detect serum antibodies against *Borrelia burgdorferi* of the IgM and IgG classes, before and after antibiotic therapy. The study included 86 patients hospitalized in the Department of Infectious Diseases and Neuroinfections in the Medical Academy in Białystok, diagnosed with the *erythema migrans* phase of Lyme borreliosis. Examinations were carried out twice: the first at the moment of diagnosis (Trial 1), the second after 4 weeks of antibiotic therapy. The study showed that antibiotic therapy in the early phase of borreliosis does not decrease the sensitivity of PCR and that after 4 weeks of therapy (Trial 2), spirochete DNA is still detectable in most patients (45/86). There was no correlation between detectability of spirochete DNA and the presence of antibodies against *B. burgdorferi* s.l. (assessed by ELISA) during the course of *erythema migrans*. The largest percentage of positive results in the detection of *B. burgdorferi* s.l. DNA was observed in patients who simultaneously possessed IgM and IgG antibodies against *B. burgdorferi*, while the lowest percentage of PCR positive results was among patients with only IgM antibodies.

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INTRODUCTION

The number of diagnosed cases of borreliosis (Lyme disease) is increasing rapidly in Poland, especially in the northeastern part of the country. *Erythema migrans*, an early phase of the disease affecting the skin, is diagnosed mainly through epidemiological interview and the characteristic alterations it leaves on the skin of patients. The diagnosis of Lyme borreliosis, particularly its arthritic, neurological and cardiological forms, is still difficult for clinicians. Basic serological/immunoenzymatic methodology (ELISA) is inadequate in the case of *erythema migrans* because the immunological reaction, i.e. the presence of specific antibodies in the serum, does not occur until 6-8 weeks after infection when the skin rash begins to recede. In rare cases, antibodies against *Borrelia burgdorferi* appear even 10 weeks after early antibiotic administration. In addition, the results obtained from serological methodology

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often have ambiguous interpretations, e.g. manufacturers use various strains of spirochetes from different genospecies. Therefore, the development of a comparable methodology for use in diagnostics is warranted. To date, assessments of PCR-based methods for *Borrelia burgdorferi* detection have been rather unenthusiastic. PCR methodology seems most useful in the early skin phase of the disease. It is possible to detect the spirochete in biopsies from altered areas of skin. *Erythema migrans* located at the site of the tick bite is often accompanied by multisystemic symptoms such as fever, arthritis, muscle and headache. Much evidence points to the association of early skin symptoms and bacteremia – spirochetemia, which according to Steere [13] may cause these syndromes. Wormser *et al.* [15] cultured spirochetes from peripheral blood from 50% of patients with *Erythema migrans*.

The aim of the study was to assess the frequency of *Borrelia burgdorferi* DNA detection in the blood and urine of patients diagnosed with *erythema migrans*, and compare the results of PCR-based methods with the ELISA methodology.

The latter was used to detect serum antibodies against *Borrelia burgdorferi* of the IgM and IgG classes, before and after antibiotic therapy.

MATERIALS AND METHODS

The study included 86 patients hospitalized in the Department of Infectious Diseases and Neuroinfection in the Medical Academy in Białystok, diagnosed with the *Erythema migrans* phase of Lyme borreliosis. The study group was composed of 52 women and 34 men with a mean age of 48 years (range 17-71 years). Examinations were carried out twice: at the moment of diagnosis (Trial 1), i.e. 2-3 days after the skin lesion appeared, or 6-7 days after tick bite; than after 4 weeks of antibiotic therapy (doxycycline or amoxycycline) (Trial 2). A group of 14 individuals was selected that had begun antibiotic therapy before the first examination (for 2-3 days), prescribed by a general practitioner.

All patients were subjected to serological examination by the immunoenzymatic ELISA method by using the Recombinant-Borrelia IgM and IgG tests (Biomedica). Simultaneously, blood and urine were sampled for PCR. The molecular assay was conducted with primers differentiating between three genospecies of *Borrelia burgdorferi*: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*.

***Borrelia burgdorferi* sensu lato DNA detection using polymerase chain reaction (PCR).** The DNA of bacteria from human blood and urine was isolated according to the MasterPure™ DNA Purification Kit (EPICENTRE, Madison, Wisconsin, USA) and stored at -70ºC.

Nested PCR conditions. A fragment of the rrs gene, which encodes for the 16S rRNA of the small ribisomal subunit, was used for *B. burgdorferi* s.l. DNA detection. Four primer sets were designed by Marconi and Garon [6] for specific amplification of *B. burgdorferi* s.l. and three genospecies: *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*.

The 20 µl PCR mixture contained 0.5 U of Taq DNA polymerase (QIAGEN, GmbH, Hilden, Germany), 1 x reaction buffer with 1.5 mM of $MgCl_2$, 75 µl of each deoxynucleotide triphosphate (Polgen, Poland), 20 pmol of each of the two primers (SC, BB, VS and VS, respectively) and 2 µl of DNA isolated from human blood or urine. The DNA of the Bo-148c/2 strain of *B. burgdorferi* s.s. was used as a positive control. TE buffer (pH 8.0) was applied for the negative control. PCR was performed in a T-gradient thermal cycler (Biometria, Germany) and Peltier Thermal Cycler 200 (MJ Research Inc., USA). Templates were subjected to an initial denaturation step of 95ºC for 3 min, followed by 35 cycles consisting of 94ºC for 30 s, 50ºC for 45 s and 72ºC for 1 min.

The PCR products were separated on 2% agarose gel (ICN, USA) with the addition of ethidium bromide (Sigma-Aldrich, Germany) at 90 V for 45 minutes. The MW501 mass marker (Polgen, Poland) was applied for evaluation of the mass of the obtained product. The first PCR product had a size of approximately 669 base pairs (bp), and the second PCR resulted in an amplicon of 590 bp. The results of the PCR were viewed under UV light and were archived in computer storage using BioCapt software (Vilber Lourmat, France).

The results were statistically analysed using the Chi^2 and two frequencies tests in the Statistica software package.

RESULTS

In the first experimental trial, specific *B. burgdorferi* DNA was PCR-amplified in 63 patients (73.3%) among 86 diagnosed with *eythema migrans*. In the second experimental trial, 45 patients (52.3%) were positive for spirochete DNA. There was no statistical association between positive results in the 2 trials. However, in 33 cases, spirochete DNA was still recovered in spite of 4 week antibiotic therapy. Further, 12 patients were diagnosed with spirochete DNA in the second trial although results from the first were negative (Tab. 1). Urine samples were negative for *B. burgdorferi* DNA in all 86 patients in both trials.

In the group of 14 individuals that had started antibiotic therapy 4-5 days before clinical trials took place, 12 were positive (85.7%) in the first trial and 8 (57.1%) in the second after further antibiotic treatment.

Among 63 positive PCR results in Trial 1-5 were classified as *B. afzelii* (8%), all others as *B. burgdorferi* sensu stricto 58 (92%). *Borrelia garinii* DNA was not detected. In the trial where there were 45 positive PCR results, 2 (4%) were classified as *B. afzeli* 43 (96%) as *B. burgdorferi* sensu stricto. DNA of *B. garini* was not detected in Trial 2 (Tab. 4).

The ELISA method showed a significant increase of patients (37 individuals) in the second trial (after 4 week

Table 1. Detection of IgM and G antibodies against *B. burgdorferi* s.l. among PCR positive patients in the first trial.

PCR	antibodies present ELISA (+) n (%)				antibodies absent ELISA (-) n (%)	Total n (%)
	general	IgM	IgG	IgM + IgG		
PCR (+) (N = 63)	27 (73.0)	9 (60.0)	8 (72.7)	10 (90.9)	36 (73.5)	63 (73.3)
PCR (-) (N = 23)	10 (27.0)	6 (40.0)	3 (27.3)	1 (9.1)	13 (26.5)	23 (26.7)
Total (N = 86)	37 (43.0)	15 (17.4)	11 (12.8)	11 (12.8)	49 (57)	86 (100)

Table 2. Detection of IgM and G antibodies against *B. burgdorferi* s.l. amongst PCR positive patients in the second trial.

PCR	antibodies present ELISA (+) n (%)				antibodies absent ELISA (-) n (%)	Total n (%)
	general	IgM	IgG	IgM + IgG		
PCR (+) (N = 45)	25 (52.1)	8 (36.4)	7 (63.6)	10 (66.7)	20 (52.6)	45 (52.3)
PCR (-) (N = 41)	23 (47.9)	13 (63.6)	5 (36.4)	5 (33.3)	18 (47.4)	41 (47.7)
Total (N = 86)	48 (55.8)	22 (25.6)	11 (12.8)	15 (17.4)	38 (44.2)	86 (100)

therapy) with class IgM antibodies against *B. burgdorferi* as compared to the first trial (26 individuals). Similarly, there was an increase in positive results in the group of patients with class IgG antibodies in the second trial, i.e. from 22 to 26. The number of patients with *Erythema migrans* but negative for *B. burgdorferi* antibodies decreased from 38 to 49 (Tab. 3).

The 63 PCR positive patients in the first trial were divided into 4 groups according to the results of the serological ELISA method. The first group consisted of patients with class IgM antibodies against *B. burgdorferi* in their serum (9/60.0%), the second with IgG antibodies (8/72.7%), the third with both classes of antibodies (10/90.9%), and the fourth in which antibodies were not detected (36/73.5%) (Tab. 1).

There was a statistically significant association between positive PCR results and the presence of IgG antibodies against *B. burgdorferi* ($p<0.043$), and the simultaneous detection of IgM and IgG antibodies ($p<0.0002$). Among the 86 patients with *Erythema migrans*, antibodies were not detected in 49 individuals in the first trial. However, 36 individuals (73.5%) were found to possess spirochete DNA (Tab. 1).

Analogously, the second trial revealed that 8 PCR positive patients (36.4%) also possessed class IgM antibodies, 7 had IgG antibodies (63.6%), and 10 had both classes (66.7%). 38 patients in the second trial did not harbour antibodies against *B. burgdorferi*. Spirochete DNA was detected in a total of 20 patients (52.6%) of this group. There was no statistically significant association between positive PCR results and IgM/IgG antibodies in the second trial (Tab. 2).

A similar analysis in the group with 14 patients that had started therapy before trials revealed 3 patients each with IgM or IgG antibodies (21.4% each) in the first trial. After antibiotic therapy the number of patients with IgM

Table 3. The frequency of *B. burgdorferi* s.l. DNA and antibodies against *B. burgdorferi* in 86 patients diagnosed with *erythema migrans*.

Assay	Trial 1 n (%)	Trial 2 n (%)	statistically significant difference
PCR (urine)	0 (0)	0 (0)	
PCR (blood)	63 (73.3)	45 (52.3)	Ns
ELISA IgM (serum)	26 (30.2)	37 (43.0)	p<0.0000
ELISA IgG (serum)	22 (25.6)	26 (30.2)	p<0.0000
negative serology	49 (57)	38 (44.2)	Ns

Ns – Non-significant statistically

Table 4. Molecular (PCR) differentiation between genospecies of *Borrelia burgdorferi* in both trials.

	Trial 1 n (%)	Trail 2 n (%)
B. burgdorferi sensu stricto	58 (92)	43 (96)
B. afzeli	5 (8)	2 (4)
B. garini	0 (0)	0 (0)
Total	63 (100)	45 (100)

antibodies increased to 5 (35.7%), while the number with IgG antibodies remained the same. No patients in this group possessed both classes of antibodies.

DISCUSSION

PCR-based methods are receiving much attention in the diagnostics of infectious diseases because of their ability to directly detect and precisely differentiate microorganisms, in addition to their remarkable sensitivity. The clinical value of *B. burgdorferi* DNA detection in the diagnostics of early borreliosis has not been adequately addressed up to now [1, 3, 7].

In contrast to culture-based methodology, PCR amplification risks false positives on account of contamination (lesser specificity that, however, can be remedied by strict laboratory procedure) [7]. According to Bukinis and Barbour [1], this method allows for the detection of dead spirochetes, which increases sensitivity, but simultaneously precludes corroboration of active infection [1].

The efficacy of PCR methods in detection of DNA of various genospecies (*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*) in different biological samples (blood, cerebrospinal fluid, joint fluid, skin biopsies) has been confirmed [1, 9, 16].

Schwartz *et al.* [12] have assessed the sensitivity of PCR on skin biopsies from patients with *erythema migrans* as 59% compared to 57% from spirochete culture; however, *B. burgdorferi* DNA was also recovered from patients during antibiotic therapy [12]. In our experimental work, spirochete DNA was found in the blood of 85.7% of 14 patients that were in their 4^{th}-5^{th} day of therapy. After antibiotic therapy lasting 4 weeks, we still detected borrelia DNA in 57.1% of patients from this group, and 52.3% of the total number of individuals (86 patients). This large percentage questions the effectiveness of the time and dose of recommended treatment, and if antibiotics are at all adequate.

Goodmann *et al.* [2] assessed the utility of PCR for spirochetemia detection in patients with *erythema migrans* and received positive results in only 18.4% of patients (5.3% from culture); however the percentage increased in individuals with multisystemic symptoms (30.3%) and multiple skin manifestations (37.5%). In subsequent papers however, the detectability of *Borrelia* DNA improved due to superior molecular methods. Lebech *et al.* [4] obtained a 71% yield as compared to 41% from serological examination or 29% from spirochete culture. Nowakowski *et al.* [8] compared the sensitivity of various diagnostic methods in patients with *erythema migrans*. The most sensitive was PCR amplification of *B. burgdorferi* DNA from skin biopsies (80.2%). Serological examination conducted before and after treatment were characterized by 66.0% sensitivity. Cultures from skin biopsies had a much lower sensitivity (51.1%), while those from blood samples only 44.7% [8].

In our study the sensitivity of PCR was high and comparable to the previous work, 73.3% in the first trial and 52.3% in the second. Serological results using ELISA were quite similar to those from PCR after the second trial (55.8%), but much lower in the first (43%).

Oksi *et al.* [9] also assessed the diagnostic utility of PCR in patients with *erythema migrans*. Investigations of skin biopsies with PCR were positive in 35.5% of patients, while this value was 21.5% for *B. burgdorferi* cultures. In blood samples from these patients only 3.8% and 7.7% were positive in PCR amplification and cultures, respectively.

Zore *et al.* [16] also assessed the diagnostic utility of PCR in patients with *erythema migrans*. 28% of patients were positive when primers amplifying a fragment of the *fla* gene coding flagellin were used, while 61% when a fragment of the *OspA* gene was used as a diagnostic marker.

The clinical significance of *B. burgdorferi* DNA detection through PCR in urine samples is doubtful, especially since many authors have not confirmed the presence of *B. burgdorferi* in urine of patients with various clinical manifestations of Lyme borreliosis [1]. Lebech [5] determined the sensitivity of PCR in urine samples as only 13% in patients with *erythema migrans* and 7% with neuroborreliosis. Pleyer *et al.* [10] assessed the occurrence of spirochete DNA in urine from patients with a diagnosed ocular form of borreliosis. Among 6 patients with the disease, PCR amplification of *B. burgdorferi* DNA showed its presence in urine, while only 4 had antibodies in the serum as detected by ELISA. However, Western-blot analysis also gave positive results in all 6 patients. In the present study, spirochete DNA was not detected in the urine of any patients, which questions the use of this methodology.

Tylewska-Wierzbanowska [14] compared the diagnostic effectiveness of serological methods and PCR and came to the conclusion that PCR positive samples of blood, cerebrospinal fluid and joint fluid usually showed a low concentration or lack of antibodies against *B. burgdorferi* in their serum. In our study, the presence of spirochete DNA through PCR in the group of patients that did not test positive when ELISA was applied was confirmed in 73.5% of patients in trial 1 and 52.6% of trial 2. In the group with class IgG antibodies a similar number of positive instances was recorded through PCR – 72.7% of patients in Trial 1 and 63.6% of Trial 2. In the group with both classes of antibodies against *B. burgdorferi*, the percentage of PCR positive samples was 90% in Trial 1 and 66.7% in Trial 2.

Factors that could decrease the sensitivity of PCR-based methods in borreliosis diagnostics are the intraspecific variation of *B. burgdorferi* s.l. (difficulties in choosing a sequence that is species-specific and shows no intraspecific variation) and the periodical occurrence of spirochetes in bodily fluids, often in limited numbers. Therefore, a negative PCR result cannot rule out Borrelia infection, and this method is also not recommended for screening studies [1]. Further limitations include the DNA degradation during transportation and processing of samples [1].

According to Bunikis and Barbour [1], despite these technical limitations, the lack of official diagnostic kits

and high cost, PCR-based methods may have application in Lyme borreliosis in certain instances, e.g. in examining joint fluid of patients suspected of Lyme arthritis and without improvement after antibiotic treatment, or in the case of biopsies from skin rashes in persons without exposition to ticks in endemic areas [1].

CONCLUSIONS

1. Antibiotic therapy in the early phase of borreliosis (patients with EM) does not decrease the sensitivity of PCR. In a group of 14 patients that started therapy 4-5 days before clinical trials, spirochete DNA was detected in 12 (85.7%).
2. There is no correlation between detectability of spirochete DNA and the presence of antibodies against *B. burgdorferi* s.l. during the course of *erythema migrans*.
3. The urine of patients with *erythema migrans* does not seem suitable as diagnostic material for the detection of *B. burgdorferi* DNA.

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