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GENOSPECIES OF *BORRELIA BURGDORFERI* SENSU LATO IN PATIENTS WITH ERYTHEMA MIGRANS

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Abstract: *Borrelia burgdorferi* sensu lato (s.l.) complex, the etiological factor of Lyme disease, includes over a dozen species of bacteria and 3 pathogenic within it. According to many authors, the clinical symptoms of borreliosis depend on the species that cause the disease. The most frequent symptom of early localized borreliosis is erythema migrans (EM). The aim of the research was to determine species of *B. burgdorferi* s.l. in 32 patients from the Western Pomerania region in whom EM has been recognized. Blood samples of patients were investigated by PCR-RFLP method, with the use of enzyme differentiating species. The DNA of spirochetes was detected in 25 patients (25/32, 78.1%), compared with 23/32 (71.8%) of ELISA positive patients. Among 25 positive samples, 10 contained the DNA of *B. garinii* (10/25, 40%), 5 the DNA of *B. afzelii* (5/25, 20%), 4 the DNA of *B. burgdorferi* sensu stricto (s.s.) (4/25, 16%) and in 6 samples (6/25, 24%) the DNA of both *B. garinii* and *B. afzelii* was found. The DNA of *B. burgdorferi* s.l. spirochetes may be detected in patients with EM after antibiotic treatment. The most frequent species in patients with EM from the Western Pomerania region is *B. garinii*. Infections with more than one species of *B. burgdorferi* s.l. may occur in patients with EM.

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INTRODUCTION

Erythema migrans is not pathognomonic but the most common manifestation of early Lyme disease. Precise diagnosis of this rash is important in order to initiate an appropriate antibiotic treatment [18].

Despite the fact that more than twenty years have passed since the detection of the etiological factor of Lyme disease, the course of this illness, diagnostics, treatment and prognosis still present many problems. Diagnosis is complicated by seropositivity of healthy individuals, presence of antibodies after therapy and lack of humoral immune response in patients with early erythema migrans [1].

In the USA, as well as in Europe, the occurrence of *Borrelia burgdorferi* sensu lato (s.l.) spirochetes in humans and vectors of the illness – ticks from Ixodes genus are widespread. Many studies on the spreading of infection with *B. burgdorferi* s.l. in humans as well as in populations of animal reservoirs of borreliosis have been carried out in recent years, and the infection of ticks transmitting spirochetes have also been estimated [10, 13, 16, 17]. Serological ELISA techniques, indirect immunofluorescence IFA, Western blot, as well as PCR have been used in these studies. The occurrence of different species within the *B. burgdorferi* s.l. complex, having a differential tissue tropism, could decide about the character of the illness – involving joints, skin, or occupying the nervous system. There are differences in the occurrence of *B. burgdorferi* s.l. species known in the USA and in Europe, and the more frequent occurrence of Lyme arthritis of borreliosis in North

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America and dominance of neuroborreliosis and late skin forms in Europe. The early localized form of borreliosis – erythema migrans (EM) occurs in the case of infection with all species of *B. burgdorferi* s.l.

Routine diagnostics of borreliosis are based on immunoserological studies with ELISA method, and on the Western blot as the confirming test. Molecular tests do not have to be carried out routinely, but only in specific clinical situations and in specialized laboratories [6]. This study was performed to validate the PCR method and its varieties in the diagnostics of Lyme borreliosis in patients with erythema migrans after antibiotic therapy.

MATERIALS AND METHODS

A group of 32 patients were studied, 20 women and 12 men, fulfilling clinical CDC criteria for erythema migrans. The patients were diagnosed and treated in the Department for Infectious Diseases and Hepatology in Szczecin. They received an antibiotic treatment – amoxicillin 1,500–2,000 mg/d or doxicillin 200 mg/d for the 3 weeks. Then, after a period of one month, 5 ml of patients blood was collected for serological studies and for PCR. Immunoserological studies were carried out with ELISA method using recombined tests from Biomedica company.

The DNA of *B. burgdorferi* s.l. was isolated from whole blood with the use of prepared kit, MasterPure DNA Purification Kit (Epicentre, Madison, WI, USA) and kept at the temperature of -70°C untill analysis.

Gene fla encoding flagellar protein, flagelline was the marker for the detection of the DNA of *B. burgdorferi* s.l. The detection of a fragment of gene fla was carried out with nested PCR method, with the use of 2 pairs of primers: outer, 132f (5'-TGGTATGGGAGTTTCTGG-3') and 905r (5'-TCTGTCATTGTAGCATCTTT-3'), for which product of the PCR reaction had length 774 bp and inner, 220f (5'-CAGACAACAGAGGGAAAT-3') and 823r (5'-TCAAGTCTATTTTGGAAAGCACC-3'), complementary to the amplificated sequence with the use of primers 132f and 905r, and the product of this reaction had the length 604 bp. Composition of the reaction mixture and conditions of the PCR course were described earlier [19]. In order to minimize the contamination, the processes of DNA isolation, the reaction mixture preparation and electrophoresis were carried out in separate rooms.

PCR-RFLP. The amplified DNA were digested with restrictive enzyme DdeI (Q-Biogene, USA), with the use of 220f and 824r primers, in order to differentiate species from the *B. burgdorferi* s.l. complex. The application of DdeI enzyme for digesting the PCR product with mentioned primers allows to obtain restrictive patterns characteristic for: *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae* and *B. bissettii* [20]. The digestion products were separated in 3% agarosis gel and the pictures were archivized in the computer memory.

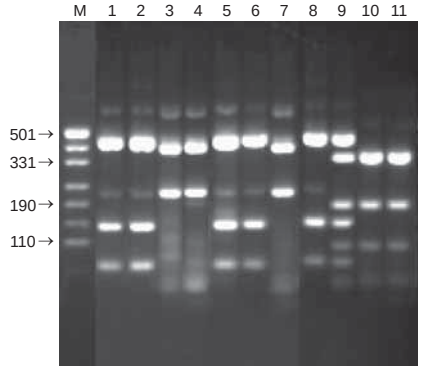
RESULTS

Among 32 blood samples from EM positive patients, the DNA of *B. burgdorferi* s.l. was found in 25 cases (78.1%). Ten of them contained the DNA of *B. garinii* (10/25, 40%), 5 the DNA of *B. afzelii* (5/25, 20%), 4 the DNA of *B. burgdorferi* s.s. (4/25, 16%) and the DNA of both *B. garinii* and *B. afzelii* was confirmed in 6 samples (6/25, 24%). To sum up, the DNA of *B. garinii* occurred in 16 blood samples and *B. afzelii* in 11 patients (Tab. 1). Figure 1 shows the different PCR-RFLP restriction patterns for *B. burgdorferi* s.l. genospecies.

A positive result of the serological study was obtained in 23 patients with EM (71.8%). The conformity in the range of positive results, i.e. the presence of DNA of *B. burgdorferi* s.l. and the presence of antibodies against antigens of *B. burgdorferi* s.l. were found in 18 samples; in 7 the serology was negative despite the presence of DNA of *B. burgdorferi*

Table 1. Results of PCR and ELISA tests for *Borrelia burgdorferi* sensu lato and genospecies in blood of patients suspected for borreliosis (F – female, M – male, bbsl – *B. burgdorferi* s.l., ba – *B. afzelii*, bg – *B. garinii*, bbss – *B. burgdorferi* s.s.).

age range (years)	sex	no of patients	PCR-(ELISA+/ ELISA-)	PCR+ (ELISA+/ELISA-)				
				bbsl (total)	ba	bg	bbss	ba+bg
> 35	F	5	2 (2/0)	3 (3/0)	1 (1/0)	2 (2/0)		
	M	4	1 (1/0)	3 (2/1)		1 (1/0)		2 (1/1)
35-50	F	6	1 (1/0)	5 (4/1)		2 (2/0)	3 (2/1)	
	M	3		3 (2/1)	2 (2/0)			1 (0/1)
< 50	F	9	3 (1/2)	6 (6/0)	2 (2/0)	2 (2/0)		2 (2/0)
	M	5		5 (3/2)	1 (1/0)	2 (1/1)	1 (1/0)	1 (0/1)
subtotal	F	20	6 (4/2)	14 (13/1)	3 (3/0)	6 (6/0)	3 (2/1)	2 (2/0)
	M	12	1 (1/0)	11 (7/4)	3 (3/0)	3 (2/1)	1 (1/0)	4 (1/3)
total	32	7 (5/2)	25 (20/5)	6 (6/0)	9 (8/1)	4 (3/1)	6 (3/3)	



M – molecular weight marker MW 501, lanes 1, 2, 5, 6, 8 – restriction patterns produced by *B. garinii*, lanes 3, 4, 7 – *B. burgdorferi* s.s., lane 9 – mixed infection of *B. garinii* and *B. afzelii*, lanes 10, 11 – *B. afzelii*.

Figure 1. DdeI restriction patterns obtained on the basis of fla gene fragment of *B. burgdorferi* s.l. strains from patients.

s.l.; in 5, despite the positive serology, the DNA of spirochete was not found, and in 2 cases, neither the DNA of spirochete nor antibodies against *B. burgdorferi* s.l. occurred in the sick persons (Tab. 1).

DISCUSSION

One of the most elementary problems in the diagnostics of borreliosis is the variety of symptoms that create a need to diversify the material taken for the purposes of investigation. This variety is also associated with different concentrations of bacteria in samples taken for analysis, and PCR results are dependent on the concentration of spirochetes in the studied samples. The sensitivity of PCR from skin bioptates taken from patients with erythema migrans, which is typical for early stage of disease and occurs at a site of a tick bite, is high enough (50–70%), but the procedure is invasive and cannot be routinely used [15]. The spirochetemia in blood likewise bacteremia in the case of other infections is a temporary state, so a high percentage of detectability is possible only in the short period of prime infection. The ability of *B. burgdorferi* to make a connection with active thrombocytes of the host cause the number of bacteria to be higher in plasma than in serum, probably because of their imprisonment during the coagulation process [4, 7]. However, the efficacy of the PCR methods in the 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 already been confirmed [2, 12, 22].

A high detectability of the DNA of *B. burgdorferi* s.l. (in 78.1% of examined people) was found in the presented study. In the literature, in patients with EM, the sensitivity of the PCR technique in the studies of skin bioptates has been at the level of 35.5%, whereas in blood samples – 3.8%. The presence of the DNA of bacteria in peripheral blood proves the spirochetemia, despite receiving antibiotic treatment. On the other hand, patients did not present any symptoms, therefore the presence of spirochetes or their fragments in the peripheral blood may be clinically asymptomatic. Perhaps the clinical consequences in the chronic form of borreliosis will be revealed in the future.

A study by Kondrusik *et al.* [8] concerned 86 patients diagnosed with erythema migrans and hospitalized in the Clinic for Infectious Diseases and Neuroinfection in the Medical Academy in Białystok. Examinations were carried out twice: the first at the moment of diagnosis, the second after 4 week antibiotic treatment. The study showed that the antibiotic therapy in the early stage of borreliosis does not decrease the sensitivity of PCR, and after 4 week therapy the DNA of spirochete is still detectable in most patients (45/86). There was no correlation between detectability of the DNA of spirochete and the presence of antibodies against *B. burgdorferi* s.l. (assessed by ELISA method) during the course of erythema migrans. The highest percentage of positive results in the detection of DNA of *B. burgdorferi* s.l. was observed in patients who had at the same time antibodies of both IgM and IgG classes, while the lowest percentage of the positive PCR results was among patients with antibodies of only IgM class.

Schwartz *et al.* [15] have assessed the percentage of the PCR sensitivity on skin bioptates taken from patients with erythema migrans as 59%, and then compared this to the result obtained from the spirochete culture (57%). However, the DNA of *B. burgdorferi* was also isolated from patients during the antibiotic therapy. In our experimental work [8], the DNA of spirochete was found in the blood of 85.7% of 14 patients who were in their 4^{th}–5^{th} day of therapy. After 4 week treatment, we were still detecting the DNA of *Borrelia* in 57.1% of patients belonging to this group, and in 52.3% of the total number of individuals (86 patients). Such a high percentage throws doubt on the effectiveness of the time of recommended treatment, its dose, and questions whether antibiotics are generally adequate.

Lebech *et al.* [9] obtained a result of 71% which compared to those of 41% from serological study or 29% from spirochete culture. Nowakowski *et al.* [11] compared the sensitivity of various diagnostic methods in patients with erythema migrans. The most sensitive was the amplification of the DNA of *B. burgdorferi* coming from skin biopsies (80.2%). The serological study carried out before and after treatment were characterized by 66% sensitivity. Cultures from skin biopsies had a much lower sensitivity (51.1%), while those from blood samples only 44.7%.

In the presented study, the most frequently detected species was a spirochete *B. garinii*, whereas *B. burgdorferi* s.s was the most rare. There was a coinfection of *B. garinii* with *B. afzelii* in some samples. This result is concurs with observations of Daemarschalck *et al.* [5] who used PCR techniques in patients with borreliosis and found an prevalence of the species *B. garinii* over the others. Other authors indicate the most frequent occurrence of the species *B. afzelii* [14].

The occurrence of double infections with different species is not rare. They have been reported among both people and ticks [3, 13, 17, 21]. It occurred in our material in 6 out of 25 patients with the DNA of spirochete. Clinical implications resulting from the multiple infection with different species from *B. burgdorferi* s.l. complex are not yet known.

Serological studies carried out after 2.5 months after a tick bite and one month after treatment, revealed the presence of antibodies in over 70% of patients. From among 32 patients with a skin lesion assessed as EM, only in 18 of them the DNA of spirochetes, as well as the antibodies (56.2%), were found in the analyzed material. Negative results of the serological studies in patients with the DNA of spirochetes may be interpreted as a delayed time of their rise, or as a lack of production towards the applied antibiotic.

Therefore, the negative results of PCR and serological studies in patients with skin lesions fulfilling the clinical criteria of EM demand bigger diagnostic caution based only on lesion appearance.

The skin lesions, erythema migrans after a tick bite, may be caused by different species of *B. burgdorferi* s.l. Knowing the species may be essential during prognosis of the disease course and modification of treatment, e.g. applying antibiotics that penetrate to CNS in the case of infection with *B. garinii* which, according to literature, is connected with central nervous system infections.

Knowing the intensity of infection in the population of ticks – vectors of the disease has essential meaning in assesion of the risk of infection after a tick bite. Knowledge about the species of *B. burgdorferi* s.l. in the populations of animal reservoirs of the disease, as well as in preventing the disease, is also very important.

CONCLUSIONS

The DNA of *Borrelia burgdorferi* s.l. can be detected in patients with erythema migrans after antibiotic therapy. Among patients with EM from the West Pomerania region, *B. garinii* is the dominant genospecies of *Borrelia burgdorferi* s.l. complex. In patients with EM there can also occur a co-infection with other genospecies.

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