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BORRELIA BURGDORFERI INFECTION AMONG FORESTRY WORKERS - ASSESSED WITH AN IMMUNOENZYMATIC METHOD (ELISA), PCR, AND CORRELATED WITH THE CLINICAL STATE OF THE PATIENTS*

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Abstract: Occurrence of borreliosis in human population is associated with possibility of contact with the biological vector of this disease - a common European tick, *Ixodes ricinus*. Therefore, the highest number of cases of Lyme disease has been recorded among forestry workers and inhabitants of wooded areas. Diagnostics of borreliosis is based on immunoserologic tests - ELISA or indirect immunofluorescence method, Western blot technique, or on increasingly popular DNA examination using the polymerase chain reaction (PCR). In the present study, where 61% of the forestry workers were seropositive, we also tried to find a correlation between the results of serological tests and PCR tests with the clinical state of the patients. Despite finding IgM antibodies in 10 persons tested, which would indicate their recent infection, no DNA of *B. burgdorferi* s.l. was detected in their blood. Also, no DNA of this bacteria was present in 8 persons with IgM and IgG antibodies. No genetic material of the bacteria was found in persons with IgG antibodies, indicating the possibility of chronic infection. The clinical data suggested past symptomatic infection (ECM), or even more often, asymptomatic infection with *B. burgdorferi*.

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INTRODUCTION

Despite the elapse of more than 25 years since the first reports on borreliosis, known also as Lyme disease, the diagnostics of this entity presents many problems. The etiological factor are spirochetes existing in a number of genospecies of which three are pathogenic to humans in Europe (*B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*). The occurrence of this disease in human populations is associated with possibilities of contact with the biological vector of borreliosis - the tick, *Ixodes ricinus*. Therefore, the highest number of cases have been detected among forestry workers or inhabitants of wooded areas.

Laboratory diagnostics on Lyme disease has not reached a satisfactory level [3, 22]. A routine diagnostics consists in serological testing for the presence of antibodies against *Borrelia burgdorferi* in the ELISA method, or is based on the indirect immunofluorescence technique, both of them verified by Western blot technique [3, 7, 33]. The tests available on the market differ in their sensitivity and specificity. Culturing of *B. burgdorferi* from the body fluids is slow and ineffective which indicates a need for

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devising a new diagnostic method [17, 22, 29]. Ever since the polymerase chain reaction (PCR) has been accepted as a sensitive method for diagnostics of microorganisms difficult for culturing, attempts have been made to apply this technique for identifying borreliosis.

Immunoserologic tests of selected populations, most often forest rangers, show a high percentage of infected persons in many regions of Poland [8, 9, 10, 31, 32]. The above-mentioned results were not always positively correlated with the clinical state of the people examined. There is an urgent need for elaborating a range of methods in order to answer the questions: whether an asymptomatic presence of antibodies against *B. burgdorferi* antigens can be a sufficient criterion for identification of borreliosis, and also whether seropositive persons, without clinical symptoms typical for borreliosis need treatment. The PCR technique for detecting the presence of the pathogen in the tissues and body fluids enables a definitive identification of *B. burgdorferi* infection.

The aim of the present study was to correlate the results of serological tests conducted with the ELISA method with the actual *B. burgdorferi* infection detected through a PCR method, and their further implications in relation to the ongoing disease process or its reoccurrence in a selected group of patients.

MATERIAL AND METHODS

The present studies were conducted based on 52 employees of the Dobrzany District - a forest administrative unit in the West Pomeranian region. The blood serum taken served later for both immunoserologic study and for PCR testing. The age of tested persons ranged from 18–64 years, mean age 38.7 yrs. There was a predominance of men in tested group - 39 (75%), while the number of women was 13 (25%). The persons tested were also questioned about past or current health problems that might have been related to *B. burgdorferi* infection, such as erythema chronicum migrans (ECM), arthritis, radiculoneuritis, cranial neuropathy, lymphocytic meningitis, or flu-like symptoms following tick bits. Also, the frequency of ticks bite cases was assessed. Clinical examination of forestry workers was carried out in the Out-patient Clinic of Infectious Diseases of Pomeranian Medical University in Szczecin. Serological ELISA tests were performed in a laboratory of the Clinic of Infectious Diseases and detection of spirochetal DNA using PCR method was conducted in the Departament of Genetics of Szczecin University.

Serological diagnosis was carried out using recombinant ELISA tests (Biotest Anti-Borrelia recombinant ELISA, AG, Germany) for detection of antibodies against *Borrelia burgdorferi* in the IgG class (against antigens: p100, OspC, p41, and p18, Osp17) and in the IgM class (against antigens: OspC and p41). The source of recombinant antigens were strains of *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*. The test sensitivity was estimated as 100% for IgG and 96% for IgM and the specificity - 84.6% for IgG and 93.8% for IgM. The results are expressed in relative quantification units (RU/ml).

Table 1. Results of immunoenzymatic study tests of the group of 52 forestry workers - including sex, mean age and serological pattern.

Positive result			Negative result
32 (61.5%)			20 (38.5%)
mean age: 39.4	IgG+/IgM	14 (26.9%)	mean age: 38.0
male: 28/39	IgG-/Ig M	10 (19.2%)	male: 11/39
female: 4/13	IgG+/IgM	8 (15.3%)	female: 9/13

Table 2. Comparison of clinical data and immunoenzymatic tests of the group of 52 forest workers.

Clinical data	Total number of persons examined n (%)	Number of seropositive persons n (%)	Number of seronegative persons n (%)
Health problems	23 (44.3)	7 (21.8)	16 (80)
ECM	4 (7.7)	3 (9.3)	1 (5)
Arthralgia	12 (24.0)	4 (12.4)	8 (40)
Radiculopathy	7 (13.5)	0 (0)	7 (35)
No health problems	29 (55.7)	25 (78.2)	4 (20)
Tick exposure	52 (100)	32 (100)	20 (100)

Detection of DNA of *Borrelia burgdorferi* sensu lato with the method of polymerase chain reaction (PCR). Five ml blood samples for detection of DNA of *B. burgdorferi* sensu lato were taken from the peripheral blood (elbow vein) on EDTA.

DNA of the bacteria was isolated from the blood samples using the method of Guy and Stanek [12]. The blood subsamples of 100 µl were merged with 200 µl of 0.7 mol ammonia water in a sterile Eppendorf test tube and boiled subsequently for 15 minutes at 100ºC, and than placed under a fume hood until the ammonia evaporated. The suspension obtained was purified according to the method described by Jaroszewski and Sawiński [1] - precipitation with 96% ethyl alcohol (500 µl) in the presence of sodium acetate at 12 000 rpm for 15 minutes. DNA was rinsed with 70% ethyl alcohol, centrifuged at 12,000 rpm for 5 minutes, dried, suspended in 40 µl TE buffer (pH 8.0) and stored at -70ºC until analysed. The PCR primers used were: SC1 (5'-GCT GUC AGT GCG TCT TAA G) and SC2 (SC1: 5'-GCT GUC AGT GCG TCT TAA G), complementary to rrs gene area encoding 16S rRNA of the small ribosome subunit of *B. burgdorferi* sensu lato [23].

The reaction mixture (20 µl) contained 0.5 U of Taq DNA polymerase (Qiagen), × 1 concentrated reaction buffer, 50 µM of each trideoxynucleotide, 400 pM of both primers SC1 and SC2, and 2 µl of DNA isolated from the blood. DNA of the strain Bo-148c/2 *B. burgdorferi* sensu stricto (kindly provided by Dr Stańczak) was used as a positive control. The above-mentioned DNA has been identified at the Loyola Medical Center, Maywood, Illinois, USA [37]. The PCR reaction was carried out in a non-oil, T-gradient thermocycler (Biometra, Germany). The course of the PCR reaction was as follows: initial

denaturation at 94ºC for 3 min. 35 cycles including denaturation at 94ºC for 30 s, incorporation of primers at 64ºC for 45 s and chain elongation at 72ºC for 45 s, and also terminal elongation at 72ºC for 7 minutes. The reaction products were separated in 2% agarose gel (ICN, USA) supplemented with ethidium bromide (Sigma-Aldrich, Germany). Mass marker MW501 (Polgen, Łódź) was used for the estimation of the size of the product obtained. The results of the PCR reaction were viewed in UV light in the presence of ethidium bromide and saved in a computer hard drive using BioCapt software (Vilber Lourmat, France) capable of reading images transmitted from a transilluminator. Presence of a band, representing expected molecular weight, in the agarose gel of the PCR reaction was interpreted as a positive result.

RESULTS

In 32 (61.5%) out of 52 serum samples studied, the presence of antibodies against *B. burgdorferi* antigens was detected using ELISA method. Fourteen (26.9%) of the 32 positive samples showed the presence of antibodies of the IgG class, ten of them (19.2%) - of IgM class, while eight others (15.3%) contained both IgG and IgM antibodies (Tab. 1). No one of the positive serum samples showed the presence of the DNA of *B. burgdorferi* s.l. The serum samples containing no antibodies against *B. burgdorferi* antigens also contained no DNA of *B. burgdorferi* s.l.

All persons studied admitted that they had been exposed many times to tick bites, sometimes over many years. They were professionally active, without health problems that would cause prolonged absence from work. Eight persons had had skin lesions in the past, similar to ECM, 12 persons complained about transient arthralgia, while seven people reported radiculopathy. Both the arthralgia and the radicular complaints were not characteristic and the persons examined tended to connect them to physical overload, low temperature exposure, or a physical trauma. The duration of symptoms was difficult to establish due to their recurrent character; mean duration of arthralgia was 5.7 years and mean duration of raduculopathy was 3.4 years. Nobody complained about a joint inflammation associated with an oedema, suffered facial nerve palsy, or meningitis.

Among the persons admitting having skin lesions in the past, six were seropositive (four persons IgM+, one person IgG+IgM+, one person IgG+). Four persons out of those complaining of joint problems were seropositive (two persons IgM+, one person IgM+IgG+, one person IgG+). No person who complained of radicular problems was tested positively against *B. burgdorferi* antibodies (Tab. 2).

DISCUSSION

First reports of borreliosis occurring in West Pomerania region date from the 1980s [16]. The frequency of cases of *B. burgdorferi* antibodies in the blood serum of people exposed to ticks in this region has been monitored in 1997 and 1999 [26, 27]. These studies revealed that 47% of the forestry workers examined had antibodies against *B. burgdorferi* in their blood serum, while healthy people with no previous contacts with ticks had no such antibodies.

The high frequency of seropositive persons employed in forests, as demonstrated by a number of studies carried out in many regions of Poland, creates serious questions addressed to physicians, health services, and the persons concerned. The principal problem has been a diversified, and not always typical, course of the disease. For instance, the ECM, the most characteristic symptom of borreliosis, may not be visible in 20–40% of infected people. Other symptoms related to joint, nervous, and circulatory systems may occur in different combinations and in most cases are not specific.

Another unsolved problem has been the way of dealing with persons currently showing no symptoms, but having serological confirmation of the infection or a positive result of PCR test.

The most important problem is the lack of uniform, dependable diagnostics of this disease. Laboratory diagnostics of borreliosis, particularly in the early phase of infection, has not been yet completely elaborated [3, 22]. A routine diagnostics consists in serological tests for the presence of antibodies against *Borrelia burgdorferi*, but sensitivity of these methods during early infection is low and the level of the antibodies after treatment declines slowly [3, 7, 33]. Culturing of *B. burgdorferi* from body fluids is slow and ineffective (long period of growth, high cost of culture, small percentage of positive inoculations) and it indicates a need for a new diagnostic method [17, 22, 29].

Since the polymerase chain reaction (PCR) has been approved as a sensitive method for diagnosing micro-organisms difficult to culture, attempts have been made to apply this technique for detecting borreliosis. Under experimental conditions, the PCR sensitivity is extremely high and it can detect a DNA matrix even from a single copy of a gene; however, the application of this method to clinical conditions still poses certain problems and requires optimisation [3, 11, 29, 34].

In an earlier study on detectability of DNA of *B. burgdorferi* s.l. in the blood of patients with borreliosis diagnosed clinically, epidemiologically, and serologically, 23 out of 30 samples studied were positive [36]. For comparing the sensitivity of the PCR test a total of three different pairs of primers complementary to three different fragments of genes were used and polymerases manufactured by two companies were employed. Despite the present work being based on the most sensitive set of reagents, determined in the paper mentioned above, all blood samples tested presently were negative for the presence of DNA of *B. burgdorferi* s.l.

Spirochetes of *Borrelia burgdorferi* reach the skin of a host during the blood meal of a tick, [6] and they subsequently migrate to various tissues, where a persistent infection is established. Such infection may not be eliminated by the immune system of the host [35]. The mechanisms triggered and maintained by the disease are

not well defined, but we know that *B. burgdorferi* is present in inflamed- and chronically infected tissues of hosts, and only occasionally in the blood [4, 5, 13, 39, 41].

Many authors emphasize the usefulness of the PCR technique in clinical diagnostics of borreliosis, stressing that even in the acute phase the sensitivity is different for different tissues and body fluids [14, 18, 21]. Oksi *et al.* [28] studied sensitivity of PCR technique in diagnostics of ECM and received a positive result in 35.5% of the skin biopsy samples but only in 3.8% of the blood samples.

While formulating the goal of this work we expected to obtain genetic material of *B. burgdorferi* in the blood serum of those infected persons, where the bacteria were dispersed from the prime inoculation site during either recent infection or an advanced stage of the disease where bacteria were released to the blood stream from other tissues and organs [15]. In the former case, no antibodies were expected or only antibodies of the IgM class. In the chronic disease, however, the blood serum should contain antibodies of the IgG class with traces of persisting IgMs. Despite that in the blood of 10 persons, antibodies of the IgM class were present, suggesting a currently occurring disease process, no DNA of *B. burgdorferi* s.l. was detected in their blood. A similar situation was observed in 8 further persons with antibodies of the classes IgM and IgG and in persons with IgG antibodies.

Also, no DNA of *B. burgdorferi* s.l. was recorded in seronegative people exposed to ticks in whom, in a case of recent infection, the PCR technique gives a chance of a sooner receipt of a positive test result than in the case of immunoenzymatic studies [24].

In view of the above-mentioned studies, interpretation of the clinical state of the tested group also raises many doubts. The forestry workers examined in the present work did not show disease symptoms of an ongoing borreliosis, the reported health problems were not characteristic and were observed in both groups: serologically negative and positive.

Persons admitting occurrence of skin lesion, that might be interpreted as ECM, receiving no treatment, did not report health problems. This may suggest the possibility of an asymptomatic infection of *B. burgdorferi* in the majority of persons examined, perhaps with elimination of the pathogens and antibodies persisting in the blood serum.

The present observations are consistent with the results of Nohlmans *et al.* [27] who, in the autumn of 1989, conducted seroepidemiological study among 440 hunters and 1,052 blood donors from different regions of the Netherlands. Among the hunters, 15% had antigens against *B. burgdorferi* of the IgG class, while the latter group studied had 9% of seropositive people. Among the seropositive hunters as much as 94% did not show any symptoms of borreliosis. Nohlmans believes that asymptomatic infections caused by *B. burgdorferi* occur in individuals frequently exposed to ticks.

Each year in Europe, North America and Asia high numbers of people and animals become infected with *B. burgdorferi* spirochetes but not all infected individuals develop clinical symptoms of the disease [20, 40]. Berglund *et al.* [2], Levy *et al.* [19], Steere *et al.* [38] estimate that among infected individuals between 5 and 50% develop clinical symptoms. It is not sure, what factors determine the course on an asymptomatic infection or an infection with symptoms, self-limiting one or chronic and progressing, although it has been demonstrated that a high number of *Borrelia* in tissues of experimentally infected mouse could trigger an inflammatory reaction [30].

In view of the present study, the negative results of the PCR tests better correlate with clinical results (absence of chronic borreliosis among people studied), than the immunoserologic diagnostics.

Summing up, in the presently used diagnostics for detection of borreliosis, particularly in its chronic phase, without initial typical manifestations like ECM, there is a need for implementation of a number of diagnostic techniques both immunoserologic ones and genetic. Their interpretation and treatment prescribed should be based on clinical data.

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