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WHAT IS THE PERCENTAGE OF PATHOGENIC BORRELIAE IN SPIROCHAETAL FINDINGS OF MOSQUITO LARVAE?

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Abstract: In this study, larvae (1,179 ex.) of mosquito genera *Culex* were examined for the presence of spirochaetes by Dark Field Microscopy (DFM) at the locality of Blansko (Czech Republic) in of 2004–2008. DFM spirochaete positive samples (25.4%) were investigated by nested PCR; only 4 samples were positive for the presence of *Borrelia burgdorferi* sensu lato, which is 0.3% of the total examined samples. We can conclude that only a low percentage of pathogenic borreliae are presented in mosquito larvae, while the spirochaete of undefined genera infect larvae in high amounts.

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INTRODUCTION

Lyme borreliosis, caused by *Borrelia burgdorferi* sensu lato, is the most frequent zoonotic multisystem disease of humans in Europe, with the mean annual incidence of up to 70 cases per 100,000 inhabitants in some of European countries [12]. People and animals become infected with these bacteria by ticks of the genus *Ixodes* [4]. Except for the presence of pathogenic borreliae in *Ixodes ricinus* ticks as the principal European species transmitting *Borrelia burgdorferi* s.l., some information about the persistence of those spirochaetes in haematophagous insects has been published [8, 12, 13, 16, 18]. Some articles describe spirochaetes in the midgut of mosquito imagoes [5, 6, 21], and some authors mention cases of Lyme borreliosis caused by insect bite [3, 17]. From physicians' reviews, these insects are introduced as being about 20% of the potential source of infection in the Czech Republic [10]. This data of the presence of pathogenic borreliae in haematophagous insects opened the discussion of further potential vectors of Lyme disease. After finding borreliae in the midgut of imago mosquitoes [2, 23, 24], we focused on the presence of *Borreliae* in the mosquito development stage – larvae of the third and fourth instar. The aim of this study was to find how high is the infection level and the percentage representation of non-defined spirochaetes in examined mosquito larvae.

MATERIAL AND METHODS

The samples were obtained in the locality of Blansko (l.r. Blansko) 30 km from Brno city, situated in the Moravian Karst, which is rich in small rodents positive for pathogenic borreliae (*B. garinii*). An area on the slope of a hill around the small Hluchov brook, covered by mixed wood, cca. 300 m above sea level, used as small allotments among orchards with fruit trees was the collecting site. Larvae were collected from rainwater barrels during the summer and autumn of 2004 and 2008. Collection dates were planned rather to late in the summer and autumn because, according to our experience, higher positivity was anticipated. A total of 33 collections containing 1,179 larvae *Culex* (*Culex*) *pipiens* sensu lato were investigated individually.

The larvae midgut was extracted and examined by dark-field microscopy as described by Žákovská *et al.* [26].

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Samples with a content of less than 50 spirochaetes were intended only for PCR reaction. When the amount was higher, two samples, one for PCR and second for the cultivation in BSK-H medium, were prepared.

Borrelia-specific DNA samples were isolated from homogenates using a DNA isolation kit QIAamp DNA Blood Mini Kit (Qiagen, Germany). DNA was eluted from QIAamp membrane in 40 µl of elution buffer. A volume of 4 µl of this prepared solution was used for amplification.

PCR assay based on the specific flagellin sequence amplification for detection of *B. burgdorferi* s.l. was performed [20]. The 40 µl of PCR mixture contained: 1x HotStarTaq Master Mix (Qiagen, Germany), 0.1 pmol of each FL3 and FL5 primers, and 20 pmol of FL6 and FL7 primers, 100 µM of dUTP (Sigma, USA), 102 internal competitive standard (Genex CZ, Czech Republic), and 4 µl of template DNA received after standard DNA isolation. All PCR runs were performed on a thermocycler (PTC-200, MJ Research) with the following profile: an initial activation step at 96°C for 12 min, 30 cycles consisting of a denaturation step for 10 sec at 96°C, an annealing step for 10 sec at 68°C, an extension step for 40 sec at 72°C, and additional 45 cycles consisting of 10 sec at 96°C of denaturation, 10 sec at 54°C of annealing, and of extension step at 72°C for 30 sec.

The resulting products of amplification were separated on 2% agarose gel containing ethidium bromide (5 µg/1 ml) and visualized using a UV illumination. In the case of a positive finding, the 276 bp – long amplification product was visible. In the case of sample negativity, only the amplification product of 420 bp was detected. No amplification product was detectable in the case of inhibition of PCR reaction.

Positive control was used both during isolation of DNA and PCR reaction.

RESULTS AND DISCUSSION

The results of the present study are shown in Table 1. All mosquitos collected in the locality Blansko, belonged only to complex *Culex* (*Culex*) *pipiens* s.l. A total of 1,179 larvae were collected in the summer (682 ex.) and in autum periods (497 ex.).

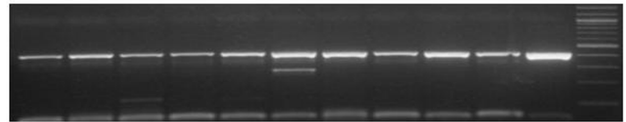

Figure 1. Agarose gel electrophoresis. Results of one-tube nested PCR amplification of flagellin gene sequence specific for *Borrelia burgdorferi* sensu lato (276 bp fragment). Negative sample shows only the product of internal control of 420 bp (samples no CX10, CX17, CX21, CB5, P48, P47, DK, P77, P32, P46), positive sample is P49 (276 bp and 420 bp).

Spirochaetal positivity was found in 299 samples (25.4%) (Tab. 1). Some collections (12/33) showed a high percentage of positivity (more than 30%), whereas 5/33 collections were negative. Among the DFM 299 positive samples, four sample were PCR positive for borreliae detected by PCR as *B. burgdorferi* s.l. (0.3%) (Tab. 1). All isolation attempts were negative.

The discovery of pathogens in blood feeding mosquito imagoes give rise to the question whether these species of insects could partially participate in the life cycle of borreliae. The question of formatting in the cycle was made more unclear by our previous finding of pathogenic borreliae (*B. garinii*) even in the mosquito larvae [25]. Using the DFM method, we found a high percentage of positive spirochaetes in submitted individual collections, and we wanted to know what is the representation of pathogenic borreliae and how much is represented by other spirochaetes. To date, several studies about the question of transmission of *B. burgdorferi*, the agent of Lyme disease, have been reported. At first, some studies described the presence of spirochaetes in haematophagous arthropods, except *Ixodes ricinus* ticks [5, 9]. With the development and availability of special molecular genetic techniques, pathogenic borreliae as a source of spirochaetes infecting blood-feeding arthropod were revealed [6, 7, 12, 13, 21]. Some borreliae were even isolated, and strains as *B. afzelii*, *B. garinii* detected [6, 7]. Moreover, medical studies present that insects form about 20% of the possible source of infected patients with Lyme disease [10]. These accumulated pieces of information could lead to the conclusion that, for example, mosquitoes could be vectors of the agent of Lyme borreliosis disease, *B. burgdorferi* s.l.

Table 1. Positivity of mosquitoes *C.* (*Culex*) *pipiens* – larvae in locality Blansko 2004–2008.

Year of collection	Locality	DFM			PCR			DFM positive	PCR positive	
		examined	positive		examined	positive				
		n	n	%	n	n	%	n	n	%
2004	Blansko	208	48	23.1	208	1	0.5	48	1	2.1
2005	Blansko	459	82	17.9	459	0	0	82	0	0
2007	Blansko	243	73	30	243	0	0	73	0	0
2008	Blansko	269	96	35.7	269	3	1.1	96	3	3.1
Total	Blansko	1,179	299	25.4	1179	4	0.3	299	4	1.3

On the contrary, in the study by Matuschka and Richter [19] the role of the mosquito as a transmitter of Lyme disease is denied. According to this study, mosquitoes fail to transmit spirochaetes to a second level of host and these authors present proof of this statement, for example, that the sucking time of a mosquito is very short compared to that of an ixodid tick. This argument can be supported by the fact that, for example, the role of mosquitoes in the transmission has not been studied experimentally. In spite of the fact that there exist many negative opinions about the role of haematophagous arthropods as a vector of LD, the detection of borreliae in blood sucking arthropods can indicate another possible source of high risk to humans. There probably exists the mechanical transmission of these pathogens. From our results taken until 2001, 3.3% of examined mosquitoe of the genera *Aëdes* spp., *Ochlerotatus* spp., *Culex* spp. were DFM positive, and of these 0.7% consisted of pathogenic borreliae (in the same mosquitoes genera). Other authors [21] have reported a positivity rate from 0.7–7.6% in *Ochlerotatus* (*Ochlerotatus*) *cantans* formally *Aëdes cantans*, *A. vexans*, *Culex pipiens* and *C. pipiens molestus*. The similar positivity of Sanogo's report fluctuated from 1.9–5.1% in the same species of mosquitoes. 1.1% of *Aëdes* spp. and 0.3% of *Culex* spp. has been reported in north-eastern Poland [22]. According to more recent Polish study, the mosquitoes of genera *Aëdes* and *Culex* were infected in 1.25% [12], while two years later, among the collected mosquitoes of the genus *Aëdes*, the prevalence of *B. burgdorferi* was 0.8% [13, 14, 15]. This low number shows the similarity also with our results. In our paper, the total spirochaetal positivity in larvae was 25.4%. Spirochaetes were detected by using DFM method in species *Culex* (*Culex*) *pipiens* s.l., which corresponds to the positivity findings of previously cited Czech authors. While this is a high percentage of spirochaetes by DFM method, this paper revealed a very low prevalence of *B. burgdorferi* 0.3% (4 samples) shown by PCR. In our previous study, made in the summer of 2001, DFM positivity of 439 *Culex* (*Culex*) *pipiens pipiens* larvae collected in the surrounding of Brno was 2.28%, and a total of 5 samples (1.14%) were positive for *B. burgdorferi* s.l. DNA [26]. The differences were probably caused by different locality, number of samples and period of collection.

There remains the question whether more than four samples could be positive. Of course, there is a possibility of obtaining a lower number of PCR positive samples because of the small number of spirochaetes by the relatively low sensitivity of single step PCR method, and by losses during the isolation process. The tendency of PCR detection to miss some of the *Borrelia* infections in ticks has already been reported [11], and this could be consistent with the findings of the present study. This observation could be explained by the presence of some inhibitors in the samples, which resulted in the lower sensitivity of the reaction [1]. Another reason for lower PCR sensitivity is reported by Hubálek and Halouzka [8] who devoted a long study of borrelia numbers in ticks, and reported that 34% of positive ticks contained less than 10 spirochaetes. That is why samples from this range are unlikely to have been picked up by our method. The tested sensitivity was about 130 borreliae in sample per reaction. In spite of all these facts there was a decidedly very low positive capture of PCR reaction, and we conclude that larvae midgut contains a very high number of undefined spirochaetes of genus other than those of borreliae.

From these results we cannot consider mosquito larvae to be a suitable milieu for borreliae, but the question of how do they access them remains unclear.

Acknowledgements

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