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POSITIVE FINDINGS OF *BORRELIA BURGDORFERI* IN *CULEX* (*CULEX*) *PIPIENS PIPIENS* LARVAE IN THE SURROUNDING OF BRNO CITY DETERMINED BY THE PCR METHOD

Alena Žákovská[1], Petra Nejedlá[1], Alena Holíková[1], Miloš Dendis[2]

[1]Department of Comparative Animal Physiology and General Zoology, Faculty of Science, Masaryk University, Brno, Czech Republic
[2]Genetic Laboratory CKTCH, Brno, Czech Republic

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Abstract: After first finding *Borrelia* in the midgut of imago mosquitoes, we concentrated on the presence of *Borrelia* in mosquitoe development stages - larvae of the third or fourth instar. In the summer season in the years of 2000–2001 a total of 439 *Culex* (*Culex*) *pipiens pipiens* larvae were collected from a barrel of rainwater in the Obřany holiday area of Brno city (East Moravia, Czech Republic). The larvae midgut was observed under dark-field microscopy. Ten DFM positive samples (2.28%) were further analysed using the single-tube nested PCR method for the presence of flagellum DNA sequence specific for *Borrelia burgdorferi* sensu lato, of which 5 were positive. Borrelian positivity of *Culex* (*C.*) *pipiens pipiens* larvae was 1.14%. One spirochete isolated strain in BSK-H medium was obtained. PCR detection for borrelian DNA of the isolated strain was negative. From these results we can conclude that a low percentage *Borreliae* can be also found in mosquitoe larvae and are likely to survive into imago stage.

Address for correspondence: Alena Žákovská, Department of Comparative Animal Physiology and General Zoology, Faculty of Science, Masaryk University, Kotlářská 2, CZ-61137 Brno, Czech Republic. E-mail: alenazak@sci.muni.cz

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INTRODUCTION

Borrelia burgdorferi is a pathogenic bacterium which causes a multisystem, inflammatory disease known as Lyme disease. *Borrelia burgdorferi* sensu stricto, *Borrelia afzelii* and *Borrelia garinii* are the most prevalent species in Europe and also occur in the Czech Republic [7, 11]. Lyme disease has been monitoring since 1985 (diagnosis A 69.2) in the Czech Republic and in 2001 about 3,323 cases were cured [1].

The main vector of infectious agents in the Czech Republic is *Ixodes ricinus* (L.) tick, this being the most common tick in Europe. Ecology of the Lyme disease agent has been investigated mainly in ixodid ticks [2], but less often in other blood - sucking arthropods. According to several references [3, 4, 8, 9, 13], however, biting insects (deer flies, black flies and mosquitoes) have also been infected with borreliae. Spirochetes have also been detected in mosquitoes larvae and pupae e.g. *Anopheles maculipennis*, *Culicine*, *Culex pipiens*, *Theobaldia spathipualpis* [13]. We found some positive larvae of *Culex* (*C.*) *pipiens pipiens* with the presence of borrelian DNA in Brno - Obřany locality, also in 1999 [15] but in years of 2000–2001 we continued to examine more mosquitoe larvae for more extensive examination. The presence of these pathogenic spirochaetes in the midgut and other parts of the soma, above all in salivary glands, may include the question of the potential vector of Lyme disease and could help to explain the epidemiology of Lyme disease.

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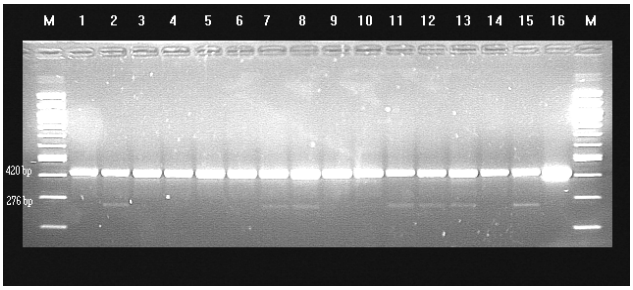

Figure 1. Agarose gel electrophoresis. Results of single-tube nested PCR amplification of flagellum gene sequence specific for *Borrelia burgdorferi* sensu lato (276 bp fragment). 420 bp fragment resulting from amplification of internal control (102 copies of MIMIC internal control ligated into the plasmid). Lane 1–4 sample from *Ixodes ricinus*, 5–13 from mosquitoe larvae. Lanes 5, 6, 9, 10 - negative samples, 7, 8, 11–13 positive samples. Lane 14 - isolation negative control (PCR mixture obtained an equivalent volume of distilled water instead of DNA added to the other samples), 15 - positive isolation control (isolation of 102 spirochetes), 16 - PCR negative control (distilled water instead of DNA). M - 100 bp ladder molecular weight standard. Samples in lanes 2, 7, 8, 11, 12 and 13 are positive.

MATERIAL AND METHOD

The locality of Brno - Obřany was chosen because it is a holiday area where many people spend much of their spare time. The locality is situated northwest part of, and 2 km from Brno city, at ca. about 350 m above sea level, on the slope of a hill. This area is used as small allotments among orchards with fruit trees. All larvae were collected from the rainwater barrel during the summer of 2001. The total of both collections contained 439 *Culex* (*C.*) *pipiens pipiens* larvae.

The larvae midgut was extracted from each sample. Its content was triturated in a drop of saline solution. Specimens were examined for the presence of spirochetes by dark - field microscopy at 400 × magnification.

In the case of spirochete content with amounts less than 100 spirochaetes in the midgut, only the sample for PCR was prepared. The midgut harboured more than 100 spirochaetes, both samples - the one for PCR and the other for the cultivation in BSK-H medium - were prepared to obtain spirochete strain for further detailed detection.

Borrelian DNA samples was isolated from homogenates using a DNA isolation kit (Malamité v.o.s., Czech Republic). This procedure is based on cell lysis by sarkosyl and chaotropic ions and subsequent binding of DNA in silica particles. DNA was eluted from silica particles in 20 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA). Volume of 5 ml of this preparation was used for amplification.

PCR assay based on specific flagellum sequence amplification for detection of *B. burgdorferi* s.l. was performed. The 50 µl PCR mixture contained 1x HotStart Master Mix (Qiagen, Germany), 15 pmol of UNF1 mixture primers, 15 pmol of each FL3 primer (mga gct tct gat gat gct gct ggy atg ggr g) and FL5 primer (grg gaa ctt gat tag cyt gyg caa tca ttg cc) [10], 100 µM of dUTP (Sigma), and 5 µl of template DNA received after standard DNA isolation. The PCR reaction was performed using a thermal cycler (PTC-200, MJ Research) under the following conditions: (a) an initial activation step at 96ºC for 12 min; (b) 30 cycles consisting of a denaturation step for 10 sec at 96ºC, an annealing step for 10 sec at 65ºC, and an extension step for 40 sec at 72ºC; and (c) the final extension was at 72ºC for 4 min.

The flagellum gene (fla) sequences of *Borrelia burgdorferi* sensu stricto (accession number: X56334), *Borrelia afzelii* (accession number: X75202) and *Borrelia garinii* (accession number: ABO17479) were gathered from GeneBank database (National Centre for Biotechnology Information - NCBI). A search for primer target sequence homology was made by the BLAST algorithm. To avoid false positive and negative results a complex of positive, negative and internal controls was used. Isolation-positive control serves for: 1. following of success of DNA isolation and 2. as well as the positive PCR control. The internal competitive standard can also serve as the positive PCR control (Fig. 1). Sensitivity of the PCR detection is about 130 spirochetes per insect sample.

Table 1. The presence of DNA of *Borrelia burgdorferi* s. l. in *Culex* (*C.*) *pipiens pipiens* larvae from 1999–2001.

Year of collection	No. of DFM positive larvae/ No. of collected larvae	Number of PCR positive (%)
1999	3/59 (5.08%)	3 (5.08%)
2000–2001	10/439 (2.28%)	5 (1.14%)
Total	13/498 (2.61%)	8 (1.61%)

RESULTS

From a total of 439 *Culex* (*C.*) *pipiens pipiens* larvae examined in 2000–2001, 10 were DFM positive for the presence of spirochete (positivity was 2.28%) of which 5 were positive for the presence of *Borrelia burgdorferi* sensu lato using single-tube nested PCR method (1.14%). A total of 13 spirochete positive samples (2.61%) was detected by DFM method; and 8 were positive in PCR reaction for the presence of *Borrelia burgdorferi* sensu lato (1.61%) in 1999–2001 in the Brno - Obřany locality (Fig. 1, Tab. 1). From the isolation attempts, 1 spirochete isolated strain in BSK-H medium was obtained. PCR detection for borrelian DNA of isolated strain was negative.

DISCUSSION

Spirochaetes has been found several times in the midgut of mosquito imagines. In some studies, spirochetes from female mosquitoes were further detected as *Borrelia burgdorferi* using various molecular methods [5, 6, 12, 15]. In the studies of Halouzka *et al.*, 1997, 1998 [4, 5] and in our as yet unpublished paper, even an isolated strain of *Borrelia afzelii* was obtained from female mosquitoes.

Spirochetes were first observed in the intestines of larvae and pupae of *Culex* (*C.*) *pipiens* s.l. by Sinton and

Shute [13]. The finding of spirochetes in larvae of *Culex* (*C.*) *pipiens* sp. in the Czech Republic in 1999 has been mentioned [15], where 3 of 59 examined larvae of *Culex* (*C.*) *pipiens pipiens* were positive for the presence of Borreliae using DFM and following PCR methods in the same locality as this study which was our first finding of Borreliae in mosquitoe larvae. In the findings for 2000–2001 presented in this paper, the number of examined larvae was 439 and DFM positivity 2.28%. PCR positivity reached only 1.14%. For both periods of examination in this locality, 498 larvae were examined and their spirochetal positivity was 2.61%, PCR positivity for the presence of Borreliae was 1.61% (Tab. 1).

With the PCR method we detected the presence of pathogenic *Borrelia burgdorferi* sensu lato in mosquitoe larvae, although if less than 130 spirochetes presented, PCR detection is negative.

The repeated findings of *Borrelia burgdorferi* in mosquitoe larvae and imago's indicate the circulation of Borreliae throughout the individual developmental stages of the mosquito. These results open the discussion about how they could get into the preimaginal stages, and if there is a possibility of transovarian transmission. So far, there have not been any positive findings of Borreliae in eggs.

The problem of relations between Borreliae, their vertebrate hosts and mosquitoes need to be studied more closely. The mode of infection of mosquitoes and their potential for Borreliae should be investigated experimentally. These questions could be answered by further investigation.

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