Biologia, Bratislava, **59**/2: 283—287, 2004 SHORT COMMUNICATION

Spirochaetes in *Culex* (*C.*) *pipiens* s.l. larvae

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ŽÁKOVSKÁ, A., JÖRKOVÁ, M., ŠERÝ, O. & DENDIS, M., Spirochaetes in *Culex* (*C.*) *pipiens* s.l. larvae. Biologia, Bratislava, **59:** 283—287, 2004; ISSN 0006-3088.

The aim of our study was to find if pathogenic spirochaete *Borrelia burgdorferi* s.l. is also present in larvae of mosquitoes and what is the level of its positivity. During August and September 2001, a total of 950 Culicine mosquito larvae of one species *Culex* (*C.*) *pipiens* s.l. were collected in Brno – Žebětín, Czech Republic, and examined by dark field microscopy (DFM) for the presence of spirochaetes. These were detected in 13 samples, which represents 1.37% of all mosquito samples. Further accurate detection was characterized by two methods: one tube nested PCR and PCR-RFLP methods were used for finding DNA of pathogenic *Borrelia afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto. One of the 13 DFM positive samples was detected as *Borrelia garinii*, i.e. 0.1% positivity of all larvae and 7.7% of DFM positive larvae. This is the third positive finding of pathogenic *Borrelia burgdorferi* s.l. in mosquito larvae.

Key words: *Culex* (*C.*) pipiens s.l., *Borrelia burgdorferi* s.l., spirochaetes, PCR, Czech Republic.

Introduction

Spirochaetes are responsible for many serious infectious diseases. One of them is Lyme disease. *Borrelia burgdorferi* of sensu lato group, an etiologic agent of Lyme disease, is transmitted to mammals in Europe primarily by the ixodid tick *Ixodes ricinus* L., 1758 (JOHNSON et al., 1984; STÜNZNER et al., 1998). The latest discoveries confirm that spirochetes are also found in other blood-sucking arthropods (mosquitoes, deer and black flies, tabanid flies, fleas and lice) (MAGNARELLI et al., 1986; DOBY et al., 1987; STANCZAK et al., 1995; HUBÁLEK et al., 1998; HALOUZKA et al., 1997, 1998, 1999; KOSIK & BOGACKA et al., 2002). These insects might play an important role in the ecology and the epidemiology of Lyme borreliosis (HALOUZKA et al., 1998, 1999). This study demonstrates the presence of borreliae in mosquito larvae of the genus *Culex*, found by the DFM method and subsequently twice verified; the isolated strain was characterized by two methods: one tube nested PCR and PCR-RFLP method (ALTSCHUL et al., 1990)

Material and methods

During August and September 2001, a total of 950 larvae of *Culex (C.) pipiens* s.l. of all four instars were collected from a pond at Brno – Žebětín by using a beaker. The locality Brno – Žebětín is situated in the west part of Brno city (49°12′ N, 16°29′ E; in the edge of forester district Podkomory). It is a part of Brněnská kotlina basin. The larvae were collected from the

rest of pond and wet ground near confluence of "Žebětínský" and "Vrbovecký" stream.

Larval abdominal content was examined and triturated on slides in a drop of phosphate-buffered saline and detected for the presence of spirochaetes by dark field microscopy (DFM) at a 200× magnification. The intensity of spirochaete infection in individual mosquito larvae was evaluated by counting and the approximate number of spirochaetes was ranked: low – up to nine spirochetes; medium – from 10 to 100; high – more than 100 per five larvae. From the medium and highly infected specimens (more than 100 spirochaetes) larvae were immediately inoculated into BSK–H medium (Sigma) for isolation attempts.

All dark-field positive samples were PCR tested independently in two laboratories by two methods:

Single-tube nested PCR
DNA was isolated from 13 DFM positive samples using a DNA isolation kit UltraClean Tissue DNA kit (MoBio, USA). Single-tube nested PCR based on specific flagellum sequence amplification for detection of *Borrelia burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* was performed (standardised PCR kit – EliGene Borrelia, ELISABETH PHARMACON, Czech Republic). Primers and PCR conditions were described previously (Picken et al., 1996). PCR product of specific length 276 bp was compared to the length of positive PCR product control in 2% agarose gel stained with ethidium bromide (Fig. 2). Positive results mean the presence of *B. burgdorferi* s.s., *B afzelii* or *B. garinii* in the sample.

Polymerase chain reaction (PCR) with restriction fragment length polymorphism (RFLP)
DNA purification. DNA of detected samples was isolated from homogenates using a DNA isolation kit (Malamité v.o.s., Czech Republic). This procedure is based on cell lyses by sarkosyl and chaotropic ions and subsequent binding of DNA in silica particles. DNA was eluted from silica particles in 20 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA). Five μl 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 1 × HotStart Master Mix (Qiagen, Germany), 15 pmol of each primers; 15 pmol of FL3 primer (5′-mga gct tct gat gat gct gct ggy atg ggr g-3′) and FL5 primer (5′-grg gaa ctt gat tag cyt gyg caa tca ttg cc-3′), 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 96 °C for 12 min; (b) thirty cycles consisting of a denaturation step for 10 s at 96 °C, an annealing step for 10 s at 65 °C, and an extension step for 40 s at 72 °C; and (c) the final extension at 72 °C for 4 min.

Restriction analysis of amplified PCR products was performed by *AluI* endonuclease digestion (New England BioLabs). The restriction fragments were separated on a 3% (wt/vol) agarose gel, stained with ethidium bromide, visualized by UV transillumination (312 nm), and analyzed by ULTRA LUM (Ultra-lum, Inc.) gel detection and analysis system. For identification of *B. burgdorferi* s. l. strains only fragments longer than 100 bp were taken into account.

Computer sequence analysis. 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 by the BLAST algorithm was made (Altschul et al., 1990). The multiple alignments by the CLUSTALW program were done (Thomson et al., 1994). The RFLP patterns, obtained after *AluI* endonuclease digestion, were predicted using the WEBCUTTER ver. 2.0. program (Fig. 3).

Prevention of false positive and false negative results
The system of positive and negative controls, internal competitive standard and uracyl DNA glycosylase to control false positive or negative results were used. Isolation of DNA from samples, PCR and detection of amplified products were performed in three different rooms.

Control of false positive results. As isolation-negative control sterile water was used. As a PCR negative control sterile TE buffer was added into the reaction. To avoid possible false-negative results due to the presence of PCR inhibitors in the samples, the internal competitive standard was used.

Control of false negative results. As a positive PCR control the amount of 10^2 copies of plasmid construct with a borrelia flagellum gene was used.

As an isolation-positive control a suspension of *B. burgdorferi* cells (6×10^2 cells ml^{-1}) was isolated with ever group of samples and the same amount of isolate was added to the PCR.

To prevent contamination by amplicons from previous reaction, dUTP and uracyl DNA glycosyl- ase were used in each reaction.

Results and discussion

From 950 *Culex* (*C.*) *pipiens* s.l. larvae collected at Brno – Žebětín spirochete positivity was found in 13 samples (1.37%) of 190 using DFM. Among the 13 positive samples just one sample (0.1%) was PCR positive for borreliae (Tabs 1, 3). The positive sample was first detected by PCR as *Borrelia burgdorferi* sensu lato and subsequently as *B. garinii* by PCR-RFLP (Figs 1, 2).

This is not the first PCR positive finding of the presence of pathogenic borreliae in mosquito larvae found in our laboratory. During the years of 1999–2001 altogether 1798 larvae of *Culex* (*C.*) *pipiens* s.l. were collected at three sites and subsequently examined (Tab. 3). Among all examined

Table 1. Findings of spirochaetes in mosquito larvae.

No. of positive samples	Intensity of DFM positive samples	PCR positive(+), negative(–) samples
6	Medium	–
1	Medium	+
6	High	–

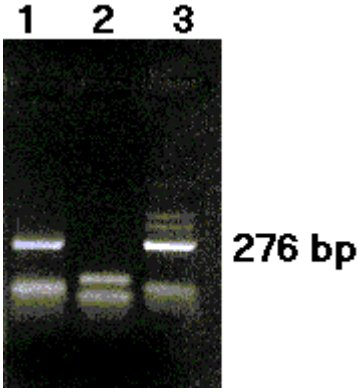

Fig. 1. Result of agarose gel electrophoresis of single-tube nested PCR amplification. 276 bp fragment results from amplification of sequence specific for *Borrelia burgdorferi sensu stricto, B. afzelii* and*B. garinii*: 1 – larvae of *Culex pipiens* s.l., positive; 2 – negative control; 3 – positive control.

larvae 27 were DFM positive for the presence of spirochaetes (1.5%), 10 were PCR positive for the presence of *B. burgdorferi* s.l. (0.6%).

Spirochaetes have already been detected in larvae and pupae of *Anopheles maculipennis* Meigen, 1818, *Culex pipiens* s.l. and *Theobaldia spathipalpis* Rondani, 1872 (Sinton & Shute, 1939; Žákovská et al., 2000). Spirochaetes have also been found in adults of *Aedes cantans* Meigen, 1818, *A. vexans* Meigen, 1830, *Culex pipiens pipiens* L., 1758, *Culex pipiens molestus* Forskal, 1775. *Aedes (Ochlerotatus) communis* De Geer, 1776 and *Aedes (Aedes) cinereus* Meigen, 1818 (Magnarelli et al., 1986; Halouzka et al., 1998; Sanogo et al., 2000; Žákovská et al., 2000). In this paper, larvae of *Culex (C.) pipiens* s.l. from Brno – Žebětín were found to be positive for the presence of spirochaetes. With the PCR – RFLP method the pathogenic *B. garinii* was detected. This confirmed the fact that *Borrelia* species can be found in mosquito larvae, though probably just in a small amount. The question is then how pathogenic *B. burgdorferi* can get into mosquito larvae. Although we did not prove the presence of spirochaetes in mosquito eggs, we can

Table 2. Occurrences of spirochaete positive mosquito larvae et different sites in the Czech Republic in 1999–2001 (Šikutová, 2001).

Site	Term of collection	DFM positive/ total	Spirochaete positivity (%)
Břeclav	Summer 1999	47/125	37.6
Valtice	Summer 1999	29/33	87.9
Břeclav	Summer 2000	0/139	0.0
Valtice	Summer 2000	131/150	87.3

Table 3. DFM and PCR positivity of *Culex pipiens* s.l. larvae for spirochaetes and *Borrelia burgdorferi* s.l.. (Žákovská et al., 2000, 2002).

Year	Site	No. of DFM positive/ no. of examined larvae	No. of PCR positive/ no. of examined larvae
1999–2001	Brno – Obřany	13/498 (2.6%)	8/498 (1.6%)
2001	Brno – Žebětín	13/950 (1.4%)	1/950 (0.1%)
2001	Vys. Mýto	1/350 (0.3%)	1/350 (0.3%)
Total		27/1798 (1.5%)	10/1798 (0.6%)
		$0.009 \leq P \leq 0.021$	$0.002 \leq P \leq 0.010$

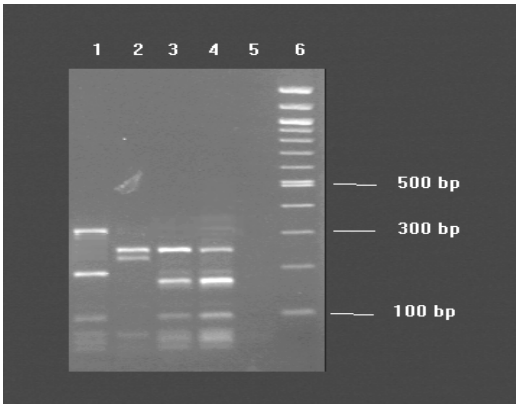

Fig. 2. Agarose gel electrophoresis. Resulting RFLP patterns obtained after *AluI* endonuclease treatment of amplified specific *Borrelia burgdorferi* s. l. flagellum sequence: lane 1 – *Borrelia burgdorferi* s.s. reference strain; 2 – *B. afzelii* reference strain; 3 – *B. garinii* reference strain; 4 – sample BRZ 10 (identified as *B. garinii*); 5 – sample BRZ 100 (negative for presence of specific *B. burgdorferi* s.l. flagellum sequence); 6 – 100 bp molecular weight standard (Malamité, CR).

consider the possibility of transovarial transmission of *B. burgdorferi*, while other non-pathogenic spirochaetes moving freely in water could get into the larvae through water filtration.

Systematic investigation of spirochaetes in mosquito larvae has not been carried out and we summarized results from other sources examined in the Czech Republic (Tab. 2). Here the high positivity in some larvae populations is incredible; at Valtice, it reached up to 87.9% and 87.3% in summer of 1999 and 2000, respectively (Šikutová, 2001). This corresponds with the results reported from the Royal Institute of Infectious Diseases in Berlin by Jaffé (1907), who observed 90% spirochaete infection in the intestine of *C. pipiens*. The results obtained in our laboratory (Tab. 3) show great differences of mosquito infection rate at individual sites examined: Brno – Obřany (2.6%), Brno – Žebětín (1.4%) and Vysoké Mýto (0.3%), with the observation of high contamination rate at Valtice, while the summer 2000 observation at Břeclav was negative. We did not manage to explain the reason of the big differences. It will probably be a matter of quantity of blood-sucking arthropods and the quality of laboratory examinations.

Ixodes ricinus ticks are the main vectors of Lyme disease in the Czech Republic. Although the presence of *B. burgdorferi* s.l. was proved even in mosquitoes, the transmission of borrelia by these insects has not yet been experimentally manifested. However, the proof of *B. burgdorferi* presence in mosquitoes could show their possible role in ecology and epidemiology of the Lyme disease.

Acknowledgements

This investigation was supported by grant: MSM 143100010 (formerly CEZ: J07/98:143100010) and by MSM 143100008.

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Received January 13, 2003
Accepted November 13, 2003

Biologia, Bratislava, **59**/2: 287–288, 2004 **Faunistical Notes**

Disparalona hamata (Birge, 1879) (Crustacea, Anomopoda) – the second record from Europe

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Disparalona hamata (Birge, 1879) (Crustacea, Branchiopoda, Anomopoda) was found in the plankton of Slovakian lowlands. This is the first record from Slovak territory and the second record from Europe. The species was recorded in the North and South America, South Asia and Africa (Smirnov, 1996). So far the only European record was reported from the Czech Republic by Šrámek-Hušek (1946), who labelled the species as *Pleuroxus striatoides* n. sp. and synonymised it with *Pleuroxus chappuisi* Brehm, 1934 in Šrámek-Hušek (1962). The author supposed that it was introduced to Europe from tropical area. Smirnov (1971) regarded the name *Pleuroxus chappuisi* as a younger synonym *Pleuroxus hamatus* Birge, 1879 (subspecies *P. hamatus hamatus* Birge, 1879). Hrbáček et al. (1978) included the species into the genus *Alonella* as *Alonella hamulata* (Birge in Limnofauna Europea). Smirnov (1996) transferred the species in the genus *Disparalona* Fryer, 1968. Nevertheless Flössner (2000) preferred again the genus *Alonella*.

Material examined: Podunajská nížina lowland – Istragovské rameno arm (47°53′ N, 17°37′ N, the Gabčíkovo village; 2.X.2003) – over 41 specimens and

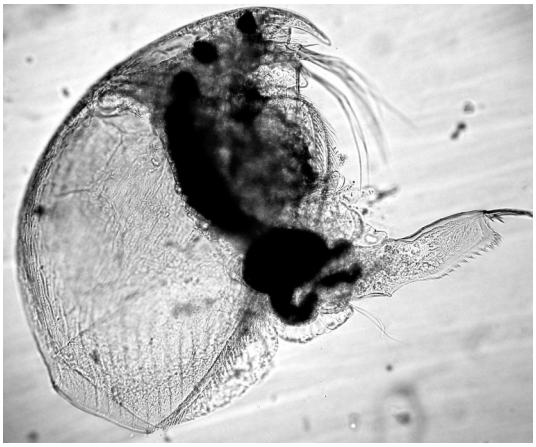

Fig. 1. *Disparalona hamata* (Birge, 1879), parthenogenetic female.

2 ephipial females (all in collection of second author). Istragov arm is a partially abandoned side arm (r. km 1815,5), situated 5 km upstream from the confluence of the tail-race canal with the old Danube.

Characteristics of Slovakian population
The body shape of mature females (Fig. 1) similar to g. *Pleuroxus*, but head is small and corresponds with genus *Disparalona* or *Alonella*. IPD: 0,8–1 it is more similar to *Alonella* and *Disparalona* than to *Pleuroxus*. Carapace with longitudinal lines near postero-ventral and antero-ventral margins. Dense short striae between lines covering all cuticular surface including head shield. Postero-ventral angle without denticles but with 7–10 short spinule-like setae (similar spinules in *Picripleuroxus laevis* (Sars, 1862) behind the posterior denticle). Shape and size of lateral antennula seta corresponds with *Pleuroxus (Peracantha) truncatus* (O. F. Müller, 1785). Spine arrangement of antenna corresponds with *Alonella* (2 long ones on distal segments and one short on 1st segment). Labral plate large triangular and blunt pointed (resemble *P. truncatus*). Postabdomen elongate and narrow resembling more *Pleuroxus denticulatus* Birge, 1879 than that of the genus *Alonella*, and never that of the European species *Disparalona rostrata* (Koch, 1841). The 1st trunk limb: IDL has one thick and heavy sclerotinized hooked seta corresponding to *Disparalona hamata*.

The second author prepares another more detailed paper with the comparison of African populations described as *Pleuroxus chappuisi*.

Acknowledgements

This study was partly supported by the Slovak Scientific Grant Agency VEGA, No. 1/8200/01.

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Received November 25, 2003
Accepted January 9, 2004