BRIEF COMMUNICATIONS AAEM

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ISOLATION OF *BORRELIA AFZELII* FROM OVERWINTERING *CULEX PIPIENS* BIOTYPE *MOLESTUS* MOSQUITOES

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Abstract: A total of 662 samples (winter period: 469; summer period: 193 specimens) of female mosquitoes of the genus *Culex*, *Aedes* and *Anopheles* were collected during the period March 2000-April 2001 from the locality of Vysoké Mýto (Eastern Bohemia, Czech Republic). They were examined by dark field microscopy for the presence of spirochetes. The motile spirochetes were observed in 4.2% of all species of investigated mosquitoes. One spirochetal strain out of the 8 isolation attempts (BRZ14) was obtained (cultivation rate was 12.5%) and the spirochetal strain was then successfully cultivated and identified using PCR for the presence of *Borrelia burgdorferi* s.l., and subsequently with the RFLP as genomospecies *Borrelia afzelii*. This strain was derived from overwintering *Culex* (*Culex*) *pipiens* biotype *molestus* female mosquitoe. This is apparently one of the sporadic cases of the occurrence of pathogenic borreliae in haematophagous arthropods, other than *Ixodes ricinus* complex ticks.

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INTRODUCTION

Spirochetes in blood-sucking arthropods have been observed since the beginning of the 20th century [10, 21, 18]. In the Czech Republic, evidence for the presence of spirochetes (including borreliae) has been reported by several authors [4, 5, 6, 7, 8, 9, 11]. The first isolated pathogenic strains of the genus *Borrelia* from *culicine* mosquitoes *Aedes vexans* and *Culex pipiens molestus* were noted in South Moravia, Czech Republic [6, 7]. Some authors, such as Hard [8], Doby [3] and Luger [14], describe clinical cases of patients reporting typical erythema migrans and antiborrelian antibodies after insect bites. The diagnosis of lyme disease in some patients was proved. These facts open the discussion of whether haematophagous insects (deer flies, black flies, biting midges and mosquitoes) play a role in the ecology and epidemiology of Lyme borreliosis [2, 3, 15].

MATERIAL AND METHODS

A total of 662 female mosquitoes (469 winter and 193 summer samples) collected in localities around the town of Vysoké Mýto (Eastern Bohemia, Czech Republic) during 2000-2001 were examined for the presence of spirochetes. Using a "Winter" aspirator, mosquitoes were collected in their typical shelters (e.g. cellars of buildings) from November-March. Summer mosquitoes were collec-

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Table 1. The occurrence of spirochaetes in mosquitoes.

Species	DFM positivity	% DFM positivity	PCR positivity	Cultivation
Summer 2000				
Culex (*C.*) *pipiens molestus*	0/1	0	0	-
Culex (*Culex*) *torrentium*	0/2	0	0	-
Ochlerotatus (*Ochlerotatus*) *communis* s. l.	7/166	4.2	0	-
Aedes (*Aedes*) *cinnereus*	0/10	0	0	-
Aedes (*Aedimorphus*) *cantans*	0/4	0	0	-
Aedes (*Aedimorphus*) *vexans*	0/7	0	0	-
Anopheles sp.	0/2	0	0	-
Culicidae g. sp.	1/1	100	0	-
Total	8/193	4.1	0	0
Winter 2000				
Culex (*C.*) *pipiens molestus*	10/200	5.0	1/200	0/4
Culex (*C.*) *pipiens pipiens*	1/17	5.9	0	0/1
Culiseta (*Neoculex*) *territans*	1/1	100	0	-
Total	12/218	5.5	1/218	0/5
Winter 2001				
Culex (*C.*) *pipiens molestus*	7/220	3.2	0	1/3
Culex (*C.*) *pipiens pipiens*	1/31	3.2	0	-
Total	8/251	3.2%	0	1/3

ted using a clap-net. The samples were kept alive under cool (5ºC) and humid (r. h. 90%) conditions until processing. Mosquitoes were dissected and their abdomen content triturated individually on a slide in a drop of saline solution. Specimens were examined for the presence of spirochetes by dark-field microscopy (DFM) at 400× magnification. The spirochetes in DFM positive samples - 28 ex., (Tab. 1.) were counted. The highly infected ones (with more than 100 spirochetes - 8 ex., (Tab. 1.)) were immediately inoculated into BSK-H medium (Sigma) supplemented with 5% rabbit serum (Sigma) and antibiotics (rifampicin 50 µg/ml, phosphomycin 100 µg/ml) for isolation attempts. Inoculated tubes were incubated at 33ºC and inspected in 5-day intervals for at least 6 weeks. Only one of isolation experiments was successful. The spirochetes tolerate the rifampicin, fosfomycin, sulfametoxazol, trimetoprin and nalidixid acid in the doses used for suppression of contamination similar to those of *Borrelia burgdorferi* s.l. The isolated strain signed as BRZ 14 was then characterized by 2 methods. One tube nested PCR [16] and by PCR-RFLP methods.

PCR

DNA was isolated from all 28 positive samples by using a DNA isolation kit Nucleospin tissue nucleospin tissues (Macherey - Nagel, Germany). Single-tube nested PCR based on specific flagellin sequence amplification for detection of *Borrelia burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* was performed. Primers and PCR conditions were described previously [19]. 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.

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). DNA purification, PCR assay and Restriction analysis steps were made as previously described in Žákovská *et al.* [24].

Computer sequence analysis. The flagelin gene (fla) sequences of *Borrelia burgdorferi* sensu stricto (Accession No.: X56334), *Borrelia afzelii* (Accession No.: X75202) and *Borrelia garinii* (Accession No.: ABO17479) were gathered from the GeneBank database (National Centre for Biotechnology Information - NCBI). A search for the primer target sequence homology was made by the BLAST algorithm [1]. Multiple alignments by the CLUSTALW programme were also performed (Thomson, Higgens and Gibson, 1994). The RFLP patterns, obtained after AluI endonuclease digestion, were predicted using the WEBCUTTER ver. 2.0. program.

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 3 different rooms.

Control of false positive results. Sterile water was used as isolation-negative control. Sterile TE buffer was added to the reaction as a PCR negative control. 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. The amount of 10^2 copies of plasmid construct with a *Borrelia flagellin* gene as a positive PCR control was used.

Isolation-positive control. A suspension of *Borrelia burgdorferi* cells (6×10^2 cells/ml) 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 glycosylase were used in each reaction.

Restriction analysis of amplified PCR products was performed by AluI endonuclease digestion (New England BioLabs). The restriction DNA fragments were analysed by agarose gel electrophoresis through 2% agarose gel, visualized by ethidium bromide staining, detected using UV transillumination (312 nm), and analysed by ULTRA LUM gel detection and analysis system (Ultra-lum, Inc.).

RESULTS

Motile spirochetes were detected in 28 of all 662 examined samples (4.2% positivity). From summer specimens (193 mosquitoes), 8 ex. were positive (4.1%), from mosquitoes collected in winter 2000 (218 mosquitoes), 12 samples were positive for the presence of spirochetes (5.5%), in winter 2001 the occurrence of spirochetes was observed in 8 samples of 251 examined mosquitoes (3.2%). Positive mosquito samples belonged to the species *Ochlerotatus* (*Ochlerotatus*) *communis* s.l., *Culex* (*Culex*) *pipiens molestus*, *C.* (*C.*) *p. pipiens*, *Culiseta* (*Neoculex*) *territans* (Tab. 1); 8 samples harboured more than 100 spirochetes (28.6%) of the positive samples, and 1.2% of all examined mosquitoes. During isolation attempts, we obtained just 1 spirochetal strain of (Tab. 1), signed BRZ 14 being cultivable in the BSK - H medium. This strain was identified as *Borrelia afzelii* using the PCR and PCR-RFLP method. The strain was isolated from overwintering *Culex* (*C.*) *pipiens* biotype *molestus* female mosquito.

DISCUSSION

The findings of spirochetes in haematophagous arthropods appeared in some works from the early beginning of the 20th century [10, 21, 18] and also later in Europe [15, 17]. The presence of spirochetes in haematophagous diptera was mostly observed in mosquitoes. Some mosquito species were examined recently in southern Moravia (Czech Republic) [6]. The authors reported a positivity rate from 0.7-7.6% in *Aedes cantans*, *A. vexans*, *Culex pipiens* and *C. p. molestus*. The similar positivity of Sanogo's report [20] fluctuated from 1.9 to 5.1% in the same species of mosquitoes. Žákovská [23] documented 1.5% of spirochete positivity from a locality in northern Moravia, Czech Republic, in the species *Ochlerotatus* (*Ochlerotatus*) *communis* s.l. and *A.* (*Aedes*) *cinereus*. Further findings by the same author were proved by examining mosquitoe larvae (*Culex* (*C.*) *pipiens* s.l.); from the total number of 498 1.6% [8] were positive for the presence of borrelian DNA [24]. Spirochetes were also detected in 1.1% of *Aedes* spp. and 0.3% of *Culex* spp. in north-eastern Poland [22]. The next 2 works proved positive for spirochetes in *Aedes* spp. collected near Szczecin in north-western Poland. Obtained prevalence was 1.25% in 2001 [12] while in a longer period, including the previous year, it was 0.8% [13]. The presence of *B. burgdorferi* in the mosquito head and digestive tract was observed after experimental infection in *Aedes aegypti*, *A. atropalpus*, *A. triseriatus* [16]; in other works *A. canadensis*, *A. stimulans* and *A. triseriatus* have also been repeatedly infected with borreliae [17]. In our paper, the total spirochetal positivity was 4.2%. Spirochetes were detected by using DFM method in species *Ochlerotatus* (*Ochlerotatus*) *communis* s. l., *Culex* (*C.*) *pipiens molestus*, *C. p. pipiens* and *Culiseta* (*Neoculex*) *territans*, which corresponds to the positivity findings of previously cited Czech authors. Spirochetes were detected also in the New World in 7-8% of *Aedes* spp. mosquitoes in Connecticut, USA [15].

The study for finding and isolating *Borrelia afzelii* - an agent of Lyme borreliosis disease in *Culex pipiens molestus* mosquito - is not the first reference for the presence of pathogenic borreliae in insects. The first isolations of *Borrelia afzelii* from mosquitoes of genus *Aedes vexans*, *Culex pipiens molestus* were announced in [5, 6], identified using PCR – RFLP method.

In this work, 2 samples were detected with PCR as *Borrelia burgdorferi* sensu lato. This is 0.3% of all examined mosquitoes (Tab. 1). One of these positive samples was successful in an isolated attempt, cultivated under laboratory conditions, and then detected using PCR – RFLP method as *Borrelia afzelii*. In our other results with mosquitoe adults [23], obtained by using PCR method, just 5 of 12 positive samples were pathogenic *Borrelia burgdorferi* s.l., which was 0.77% of all examined mosquitoes. The low incidence of pathogenic *Borrelia burgdorferi* s.l. in mosquitoes indicates that mosquitoes seem to be only sporadic carriers of the Lyme disease causative agent. Spirochetes found in most of the previous observations were not proven to be pathogenic species. So far, the transmission of *Borrelia burgdorferi* via mosquitoes has not been demonstrated, despite reports of patients. It is possible, however, that Lyme disease patients could be infected with mechanical transmission by insects.

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REFERENCES

1. Altschul SF, Gish WM, Myers EW, Lipman DJ: Basic local aligment search tool. *J Mol Biol* 1990, **215**, 403-410.

2. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwald E, Davis JP: Lyme disease – a tick-borne spirochetosis? *Science* 1982, **216**, 1317-1319.

3. Doby JM, Anderson JF, Couatarmanac'h A, Magnarelli LA Martin A: Lyme disease in Canada with possible transmission by an insect. *Zbl Bakt* 1986, **263**, 488-490.

4. Halouzka J: Borreliae in *Aedes vexans* and hibernating *Culex pipiens molestus* mosquitoes. *Biologia (Bratisl)* 1993, **48**, 123-124.

5. Halouzka J, Postic D, Hubálek Z: Isolation of *Borrelia afzelii* from mosquitoes. *Med Vet Entomol* 1997, **12**, 101-103.

6. Halouzka J, Postic D, Hubálek Z: Isolation of the spirochaete *Borrelia afzelii* from the mosquito *Aedes vexans* in the Czech Republic. *Med Vet Entomol* 1998, **12**, 103-105.

7. Halouzka J, Wilske B, Stünzner D, Sanogo YO, Hubálek Z: Isolation of *Borrelia afzelii* from overwintering *Culex pipiens* biotype molestus mosquitoes. *Infection* 1999, **27**, 275-277.

8. Hard S: Erythema chronicum migrans (*Afzelii*) associated with mosquito bite. *Acta Derm Venerol* 1966, **46**, 473-476.

9. Hubálek Z, Halouzka J, Juřicová Z: Investigation of haematophagous arthropods for borreliae - summarized data 1988-1996. *Folia Parasitol* 1998, **45**, 67-72.

10. Jaffé J: *Spirochaeta cullicis* n. sp. *Arch Protistenk* 1907, **9**, 100-107.

11. Jírovec O: Cizopasní prvoci v larvách našich muchniček. *Příroda* 1943, **26(1)**, 289-294.

12. Kosik-Bogacka D, Bukowska K, Kuźna-Grygiel W: Detection of *Borrelia burgdorferi* sensu lato in mosquitoes (*Culicidae*) in recreational areas of the city of Szczecin. *Ann Agric Environ Med* 2002, **9**, 55-57.

13. Kosik-Bogacka D, Kuźna-Grygiel W, Bukowska K: The prevalence of spirochete *Borrelia burgdorferi* sensu lato in ticks *Ixodes ricinus* and mosquitoes *Aedes* spp. Within a selected recreational area in the city of Szczecin. *Ann Agric Environ Med* 2004, **11**, 105-108.

14. Luger SW: Lyme disease transmitted by a biting fly. *N Eng J Med* 1990, **322(24)**, 1752.

15. Magnarelli LA, Anderson JF, Barbour AG: The Etiologic Agent of Lyme Disease in Deer Flies, Horse Flies, and Mosquitoes. *J Infect Dis* 1986, **154(2)**, 355-358.

16. Magnarelli LA, Freier JE, Anderson JF: Experimental infections of mosquitoes with *Borrelia burgdorferi*, the etiologic agent of Lyme disease. *J Infect Dis* 1987, **156**, 694-695.

17. Magnarelli LA, Anderson JF: Ticks and biting insects infected with the etiologic agent of Lyme disease, *Borrelia burgdorferi*. *J Clin Microbiol* 1988, **26**, 1482-1486.

18. Masseguin A, Palinacci A: Parasitisme des glandes salivaires d'un *Anopheles gambiae* par un spirochete. *Bull Soc Patho Exot* 1954, **47**, 3.

19. Picken MM, Picken RN, Han D, Cheng Y, Strle F: Single-tube nested polymerase chain reaction assay based on flagelin gene sequences for detection of *Borrelia burgdorferi* sensu lato. *Eur J Clin Microbiol Infect Dis* 1996, **15**, 486-498.

20. Sanogo YO, Halouzka J, Hubálek Z, Němec M: Detection of spirochaetes in, and isolation from, *Culicine* mosquitoes. *Folia Parasitol* 2000, **47**, 79-80.

21. Sinton JA, Shute PG: Spirochaetal infections of mosquitoes. *J Trop Med Hyg* 1939, **42**, 3-5.

22. Stanczak J, Kubica-Biernat B, Racewicz M, Kruminis-Lozowska W: Occurrence of *Borrelia spirochetes* in haematophagous insects (Diptera, Siphonaptera, Anoplura). *IX SOVE European Meeting, Prague, Czech Republic, 4-7 Sept. 1995*. Programme and Abstract, 49.

23. Žákovská A, Dendis M, Pejchalová K: Spirochaetes in *Aedes* species, *Culex pipiens pipiens* larvae and hibernating *Culex pipiens molestus* mosquitoes detected with darkfield microscopy (DFM) and polymerase chain reaction (PCR) methods. *Biologia (Bratisl)* 2000, **55(6)**, 667-670.

24. Žákovská A, Nejedlá P, Holíková A, Dendis M: Positive findings of *Borrelia burgdorferi* in *Culex* (*Culex*) *pipiens pipiens* larvae in the surrounding of Brno city determined using the PCR method. *Ann Agric Environ Med* 2002, **9**, 257-259.