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Investigation of pathogens in fleas from Lithuania

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Fleas are one of the arthropod vectors for several infectious disease causing agents. In this study we detect pathogens in fleas (*Ctenophthalmus agyrtes, Megabothris turbidus, M. walkeri, Hystrichopsylla talpae,* and *Ceratophyllus sciurorum*) collected from rodents (*Apodemus agrarius*, *Apodemus flavicollis*, *Myodes glareolus*, *Microtus agrestis*, *Microtus arvalis*, *Sciurus vulgaris*) by molecular methods. The prevalence of *B. burgdorferi* s. l. infection in rodent and 237 fleas samples was determined by polymerase chain reaction using primers SL-F and SL-R. The prevalence of *Rickettsiales* pathogens infection in fleas samples was determined by polymerase chain reaction using 16s rRNR gene primers EHR521 and EHR747. For the first time pathogens of *Rickettsiales* order were detected in fleas from Lithuania using 16s rRNR gene.

Key words: fleas, rodents, pathogens, *Borrelia burgdorferi* s. l., *Rickettsiales*

INTRODUCTION

Fleas can be found worldwide and are vectors of several important zoonoses. Diseases transmitted by fleas are of major importance to health of humans and animals globally. Multiple bacterial zoonoses overlap in their geographic distribution within wild rodent communities. Fleas are vectors of several pathogens including *Yersinia pestis*, *Rickettsia typhi*, *Rickettsia felis*, and *Bartonella henselae*, which are the etiologic agents of plague, murine typhus, flea-borne spotted fever, and cat scratch disease, respectively (de Sousa et al., 2006). *Rickettsia rickettsii,* which cause Rocky Mountain spotted fever, *Anaplasma phagocytophilum*, the etiologic agent of granulocytic anaplasmosis, and an obligate intracellular bacterium that may be carried and transmitted by fleas (Adjemian, 2008), *Borrelia burgdorferi* s. l., the etiologic agent of Lyme disease may be transmitted through tick or flea bite (Doby et al., 1991). The presence of fleas could also be significant in relation to the transmission of *B. burgdorferi* s. l. Several researchers have detected this pathogen in fleas from mammals collected worldwide (Doby et al., 1991; Lindsay et al., 1991; Teltow et al., 1991; Hubalek et al., 1998).

Fleas may also be exposed to other zoonotic rickettsial pathogens such as *Anaplasma phagocytophilum* (Adjemian, 2008; Loftis et al., 2006). Wild rodents constitute a natural reservoir of *Anaplasma* pathogen. Infected fleas with *A. phagocy-*

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tophilum have been shown in the Eastern Sierra Nevada. Adjemian et al. (2008) reported that 48 fleas from 25 rodents were co-infected with *Anaplasma* pathogens (4%). While fleas do not transmit these diseases, these results demonstrate that the host was actively infected with pathogens (Adjemian, 2008).

The aim of this study was to examine the presence of pathogens of *Rickettsiales* order and *Borrelia burgdorferi* s. l. in fleas from rodents from various sites in Lithuania.

MATERIALS AND METHODS

Flea sampling. During summer from 2006 to 2008 in Lithuania a total of 163 rodents (12 *Apodemus agrarius*, 63 *Apodemus flavicollis*, 37 *Myodes glareolus*, 9 *Microtus agrestis*, 41 *Microtus arvalis*, 1 *Sciurus vulgaris*) were caught in live traps. The trap was placed in scrub, grasslands and forests to the burrow of rodents. Trapped rodents were sacrificed by decapitation and immediately put into individual plastic bags in order not to lose fleas parasitizing on rodents. Collected fleas were stored in 70% ethanol. The structures used in flea taxonomy can only be properly studied in cleared specimens, preferably mounted on microscope slides. All fleas were identified on the basis of morphometric characteristics by Skalon (1970): 143 *Ctenophthalmus agyrtes,* 69 *Megabothris turbidus,* 25 *M. walkeri,* 18 *Hystrichopsylla talpae,* and 4 *Ceratophyllus sciurorum.* From *Apodemus agrarius* 30 fleas were collected, from *Apodemus flavicollis* – 90 fleas, from *Myodes glareolus* – 47 fleas, from *Microtus agrestis* – 28 fleas, from *Microtus arvalis* – 60 fleas and from *Sciurus vulgaris* – 4 fleas.

DNA extraction. Extraction of DNA from fleas was carried out by lyses of fleas in ammonium hydroxide (NH_4OH). 110 µl of 2.5% ammonia solution was added to the samples in microcentrifuge tubes and heated at 99 °C for 25 min in the thermostat. After a brief centrifugation the tubes were opened and heated about 15 min to evaporate ammonia (Alekseev et al., 2001).

Polymerase chain reaction (PCR). DNA from 237 fleas was tested for the presence of pathogens of *Rickettsiales*, *Anaplasma* and *B. burgdorferi* s. l.

The prevalence of *Rickettsiales* pathogen infection in fleas samples was determined by polymerase chain reaction using 16s rRNR gene primers EHR521 (5’ TGT AGG CGG TTC GGT AAG TTA AAG 3’) and EHR747 (5’ GCA CTC ATC GTT TAC AGC GTG 3’). A total volume of 3 µl extracted DNA was amplified in a 30 µl reaction mixture containing 3 µl of each primer, 15 µl 2x PCR Master Mix, and 6 µl ddH_2O. PCR products were amplified using the cycling parameters of an initial denaturation at 94 °C for 3 min, followed by 41 cycle of denaturation at 94 °C for 45 sec, annealing at 62 °C for 45 sec, and extension at 72 °C for 45 sec. Amplification was completed by holding the reaction mixture at 72 °C for 3 min to allow complete extension of PCR products. PCR products were resolved by electrophoresis in 2% agarose gel with addition of ethidium bromide and visualized under UV light. The obtained specific products of 247 base pairs were considered as a positive result.

Positive DNA results were confirmed by PCR using primers MAP4AP5 (5’ ATG AAT TAC AGA GAA TTG CTT GTA GG 3’) and MSP4AP3 (5’ TTA ATT GAA AGC AAA TCT TGC TCC TAT G 3’), which amplify 849-bp fragment of *msp4* gene from *Anaplasma* species (de la Fuente et al., 2005). Each PCR mixture consisted of the following: 2.5 µl DNA, 2 µl (10 pmol/µl) of each primer, 12.5 µl 2x PCR Master Mix (Fermentas), 6 µl ddH_2O. PCR cycles included an initial 5 min denaturation step at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec, and extension at 72 °C for 50 sec. Amplification was completed by holding the reaction mixture at 72 °C for 5 min to allow complete extension of PCR products. PCR products were resolved by 1.5% agarose gel electrophoresis with addition of ethidium bromide and visualized under UV light.

The prevalence of *B. burgdorferi* s. l. infection in rodent and flea samples was determined by polymerase chain reaction using primers SL-F (5’AATAGGTCTAATAATAGCCTTAATAGC3’) and SL-R (5’CTAGTGTTTTGCCATCTTCTT TGAAAA3’). As targets for amplification fragment of *OspA* gene (localized on lp-54 plasmid) (Fraser et al., 1997) in *B. burgdorferi* s. l. genome were used (Demaerschalck et al., 1995). A total volume of 2.5 µl extracted DNA was amplified in a 25 µl reaction mixture containing 1 µl of each primer, 12.5 µl 2x PCR Master Mix (Fermentas), and 8 µl ddH_2O. PCR products were amplified

using the cycling parameters of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 20 sec, annealing at 61 °C for 20 sec, and extension at 72 °C for 30 sec. Amplification was completed by holding the reaction mixture at 72 °C for 2 min to allow complete extension of PCR products. PCR products were resolved by 1.5% agarose gel electrophoresis with addition of ethidium bromide and visualized under UV light (EASY Win32, Herolab, Germany). The obtained specific amplification products of 307 base pairs (bp) were considered as a positive result.

RESULTS AND DISCUSSION

Infestations of fleas in small rodents were analyzed previously (Lipatova, Paulauskas, 2010). The results of this study indicated that pathogens of *Rickettsiales* order were detected in 128 of 237 fleas (54%). Pathogens of *Rickettsiales* order were detected in 62 *C. agyrtes* species fleas from 143 (43%), in 41 *M. turbidus* species fleas from 69 (59%), in 14 *H. talpae* species fleas from 18 (78%), in 10 *M. walkeri* species fleas from 25 (40%) and in 1 *C. sciurorum* species fleas from 4 (25%) (Table).

Liz et al. (2000), Christova and Gladnishka (2005), Marumoto et al. (2007), Smetanova et al. (2007) and Štefančikova et al. (2008) reported that rodents may also act as reservoir hosts of *Anaplasma* spp. Štefančikova et al. (2008) showed that in Europe *A. phagocytophilum* was detected in 6.7% of *A. agrarius* mice, and 5.6% of *M. glareolus*. Hubalek et al. (1998) showed that fleas might therefore be significant vectors of *B. burgdorferi* s. l. in the enzootic cycles among rodents. However, earlier Adjemian (2008) in Sierra Nevada found that

Table. Rodents and fleas infection of Rickettsiales and *Borrelia burgdorferi* s. l.

Rodent species (n)	Number of positive combined data (prevalence)*	Fleas species collected on different rodent species (n)	Number of fleas infected by *Rickettsiales* (n)**	Number of fleas infected by *Borrelia burgdorferi* s. l. (n)**
Apodemus agrarius (12)	2 (10.5%)	*Ctenophthalmus agyrtes* (11)	9/11	0/11
		Megabothris turbidus (13)	10/13	0/13
		Megabothris walkeri (1)	1/1	0/1
		Hystrichopsylla talpae (4)	3/4	0/4
Apodemus flavicollis (63)	8 (38.0%)	*Ctenophthalmus agyrtes* (50)	35/50	0/50
		Megabothris turbidus (20)	15/20	0/20
		Megabothris walkeri (6)	3/6	0/6
		Hystrichopsylla talpae (8)	8/8	0/8
Myodes glareolus (37)	5 (25.0%)	*Ctenophthalmus agyrtes* (22)	9/22	0/22
		Megabothris turbidus (16)	8/16	0/16
		Megabothris walkeri (3)	1/3	0/3
Microtus agrestis (9)	4 (44.0%)	*Ctenophthalmus agyrtes* (13) *Megabothris turbidus* (4)	6/13 1/4	0/13 0/4
		Megabothris walkeri (4)	3/4	0/4
		Hystrichopsylla talpae (5)	3/5	0/5
Microtus arvalis (41)	8 (57.0%)	*Ctenophthalmus agyrtes* (37)	3/37	0/37
		Megabothris turbidus (11)	7/11	0/11
		Megabothris walkeri (5)	2/5	0/5
Sciurus vulgaris (1)	0 (0.0%)	*Ceratophyllus sciurorum* (4)	1/4	0/4

* Radzijevskaja et al., 2011

** Number of infected fleas / number of collected fleas

fleas parasitizing rodents were co-infected with *R. rickettsii* and *A. phagocytophilum*. In this study the search of the etiologic agent of granulocytic anaplasmosis *A. phagocytophilum* in all infected fleas of *Rickettsiales* did not show infection with *A. phagocytophilum.*

Previous analysis showed that rodents infested by fleas were positive to *B. burgdorferi* s. l. in Lithuania (Radzijevskaja et al., 2011). Fifty seven fleas, which were collected from 18 infected rodents, were tested for the presence of *B. burgdorferi* s. l. but none of fleas was shown to be infected with *B. burgdorferi* s. l. Other European studies have reported detection of Borreliae in 8.4% of 142 fleas collected from small mammals (Hubalek et al., 1998). *B. burgdorferi* s. l. was diagnosed in flea (*Spilopsyllus cuniculi*) from foxes in northern France (Doby et al., 1991). Some studies from Texas reported the isolation of *B. burgdorferi* from one flea species (Teltow et al., 1991), and studies from Canada reported that about one of 322 fleas (*O. leucopus*) was infected by *B. burgdorferi* (Lindsay et al., 1991).

However in our study *B. burgdorferi* s. l. was not detected in fleas but fleas can play some role in transmission of pathogens of *Rickettsiales*.

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PATOGENŲ NUSTATYMAS BLUSOSE IŠ LIETUVOS

Santrauka

Blusos turi didžiulę reikšmę medicinoje kaip kelių ypač pavojingų infekcinių ligų rūšių vektorius. Tyrimo metu molekuliniais metodais nustatėme patogenus blusose, surinktose nuo graužikų. Buvo išskirta DNR iš 237 blusų. Siekiant graužikuose ir blusose aptikti *B. burgdorferi* s. l., buvo atlikta PGR pasitelkus specifinius SL-F ir SL-R pradmenis. Iš blusų išskirtoje DNR buvo ieškoma *Rickettsiales* būrio patogenų, kuriems nustatyti buvo gausinamas 16S rRNR genas.

Raktažodžiai: blusos, graužikai, patogenai, *Borrelia burgdorferi* s. l., *Rickettsiales*