ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., Suppl. 4, 2012: 111-114

Detection of Human Tick-borne Pathogens in Rodents from Bulgaria

***Iva Christova*[1], *Hristo Dimitrov*[2], *Iva Trifonova*[1], *Teodora Gladnishka*[1], *Vesela Mitkovska*[2], *Anelia Stojanova*[2], *Evgenia Taseva*[1], *Vladislava Ivanova*[1]**

[1] National Center of Infectious and Parasitic Diseases, 26 Yanko Sakazov Blvd., 1504 Sofia, Bulgaria; E-mail: iva_christova@yahoo.com
[2] Plovdiv University 'P. Hilendarski', 24 Tsar Assen Str., 4000 Plovdiv, Bulgaria; E-mail: hr_dim@abv.bg

Abstract: Rodents are important reservoirs of numerous human pathogens. A total of 284 small rodents, trapped in various regions of Bulgaria during 2011 – 2012, were examined by PCR to asses the frequency of infection with the etiological agents of Lyme borreliosis and human anaplasmosis. *Borrelia burgdorferi* DNA was detected in 64 (22.5%) of the investigated rodents. DNA of *Anaplasma phagocytophilum* was found in 25 (8.8%) of the trapped rodents. In addition, specific antibodies against *B. burgdorferi* were confirmed in 85 (29.9%) of the rodents. *Apodemus agrarius* was found to be the preferable host for *A. phagocytophilum*, while *Apodemus flavicollis* was the preferable host for *B. burgdorferi*. The prevalence of infection with *A. phagocytophilum* and *B. burgdorferi* in rodents elucidated their role in maintaining pathogens in the environment. The high frequency of the infection with both pathogens is an indicator for the risk of infection in humans.

Key words: *Lyme borreliosis, Borrelia burgdorferi, anaplasmosis, Anaplasma phagocytophilum,* PCR, ELISA

Introduction

Rodents are important reservoirs of numerous human pathogens. One of the most common groups of them is transmitted by ticks from rodents to humans.

Lyme borreliosis is the most common tick-borne infection in Bulgaria as in the whole Northern hemisphere. Several species of genus *Borrelia* can cause Lyme disease in Europe, but the main species are *Borrelia afzelii, Borrelia garinii* and *Borrelia burgdorferi* sensu stricto. Early Lyme borreliosis may appear as erythema migrans and/or fever, headache, fatigue, pain in muscles and joints. Left untreated, later symptoms include arthritis, meningitis, neuritis or skin lesions.

Borrelia is transmitted to humans by the bite of infected ticks belonging to a few species of the genus *Ixodes.* Rodents are common hosts of the ticks and serve as efficient reservoir of borreliae. Various rodent species have been implicated as natural reservoirs for *Borrelia burgdorferi* sensu lato (Aeschlimann *et al.* 1986, Matuschka *et al.* 1994, 1997).

The tick vectors of *Anaplasma phagocytophilum*, the etiological agent of human granulocytic anaplasmosis (HGA), are also *Ixodes* ticks and coinfections in patients have been documented (Mitchell *et al.* 1996). Small mammals, being common hosts of *Ixodes* ticks, are suspected of being natural reservoirs for *A. phagocytophilum*.

Human anaplasmosis is a febrile illness, which typical symptoms, beside fever, include headache, chills, and muscle aches. Usually, these symptoms occur within 1-2 weeks after the tick bite. The dis-

ease usually has favourable outcome but in immuno compromised patients can lead to fatal outcome.

The aim of this study was to assess role of the rodents as reservoirs for borreliae and anaplasmae through investigation of distribution and rate of infection of rodents trapped in different regions of Bulgaria. Rodents were captured and their tissues tested by PCR to detect infection with *B. burgdorferi* and *A. phagocytophilum.*

Materials and Methods

Samples collection

A total of 284 small rodents were trapped in numerous regions of Bulgaria during 2011 – 2012. Animals were collected by live traps, placed in outbuildings, gardens, and fields. Captured rodents were identified to species level and euthanized. Blood samples and spleen samples were collected and stored at -20 ^{0}C until use.

DNA purification

DNA was extracted from animal tissue with GenomicPrep Cells and Tissue Isolation Kits (Amersham Bioscience, UK). The protocol used was that suggested by the manufacturer. Extracted DNA was eluted in 100 µL of hydration solution.

PCR amplification

For detection of *B. burgdorferi*, reaction mixture used 5 µL DNA extraction sample as the template in a total volume of 25 µL. The reaction mixture (pure Taq Ready-To-Go PCR Beads, Amersham Bioscience, UK) contained 2.5 units of pure Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dATP, dCTP, dGTP, and dTTP. The primer pair was BBSCH1 and BBSCH2, amplifying a 400 kb fragment of the flagellin gene of *B. burgdorferi* (Schmidt *et al.* 1996). Amplified products were analyzed by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide.

For detection of *A. phagocytophilum*, a similar reaction mixture was used with primers LA1 and La6, amplifying the *epank1* gene of *A. phagocytophilum* under conditions as described by Walls *et al.* (2000). Amplified products were visualized in 1.5% agarose gel elestrophoresis.

ELISA

Blood samples from the rodents were investigated for the presence of anti-*Borrelia* antibodies by in-house ELISA test. Briefly, sonicated *B. burgdorferi* cells were coated overnight at 4 ^{0}C on the wells of 96-well plates for ELISA. Blood samples were diluted 1:100 in PBS-Tween 20-BSA and incubated on the wells for 1 h at room temperature. After washing step, anti-mouse IgG antibodies were diluted 1:800 and incubated on the wells for additional 1h at room temperature. Antigen-antibody reaction was visualized by 0.4 mg/mL OPD and 0.01% hydrogen dioxide for 30 min in a dark place. Colour reaction was read spectrophotometrically at 492 nm. Blood samples from healthy laboratory mice serve as negative controls. The cut-off value was calculated as 3 SD above mean value of the negative controls.

Results

A total of 284 small rodents were trapped in different regions of Bulgaria. Blood samples from the rodents were tested by ELISA to detect antibodies against *B. burgdorferi*, the etiological agent of Lyme borreliosis, and spleen samples from the rodents were tested by PCR to detect infection with *B. burgdorferi* and *A. phagocytophilum,* the agent of unspecific febrile human illness named anaplasmosis.

B. burgdorferi DNA was detected in 64 (22.5%) of the investigated rodents. Species identification of the infected rodents revealed that belonged to species *Apodemus flavicollis*, *Apodemus agrarius* and *Apodemus sylvaticus*. Active *Borrelia* infection was confirmed by detection of *Borrelia* DNA in 41 (32%) of the 128 trapped *A. flavicollis*, in 20 (20.8%) of the 96 investigated *A. agrarius* and in 3 (27.3%) of the 11 trapped *A. sylvaticus*. The most often infected with borreliae rodent species was *A. flavicollis* (Fig. 1).

Specific antibodies against *B. burgdorferi* were detected in higher proportion of the investigated rodents: 49 (38.3%) of the trapped *A. flavicollis*, 32 (33.3%) of the investigated *A. agrarius* and 4 (36.43%) of the trapped *A. sylvaticus* (Fig. 1).

Infection with *A. phagocytophilum* was tested only by detection of the specific bacterial DNA. Active infection with *A. phagocytophilum* was detected in lower proportion of rodents than *Borrelia* and species distribution was different. *A. phagocytophilum* DNA was detected in 11 (8.6%) of the investigated *A. flavicollis*, in 13 (13.5%) of *A. agrarius* and in 1 (9.0%) of *A. sylvaticus*. The most widely infected with anaplasmae rodent species was *A. agrarius.*

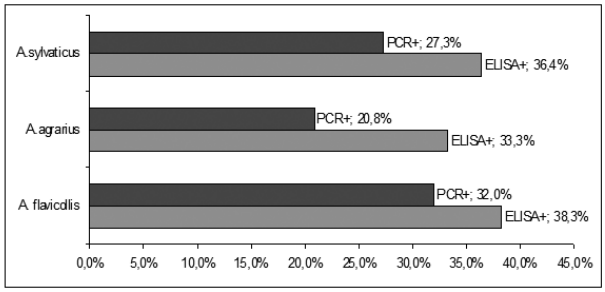

Fig. 1. Detection of *Borrelia* infection by PCR and ELISA in small rodents in Bulgaria.

Discussion

This report showed competence of three rodent species, *A. flavicollis*, *A. sylvaticus* and *A. agrarius,* as reservoirs of infection with *B. burgdorferi* and *A. phagocytophilum*, the etiological agents of Lyme borreliosis and human granulocytic anaplasmosis respectively.

Lyme borreliosis is well-known in Bulgaria with about 1000 registered new cases per year. Anaplasmosis is only occasionally detected in Bulgaria. However, our results showed that infection with both pathogens is quite common in small mammals from various regions in Bulgaria. In fact, infection with *A. phagocytophilum* was detected relatively less often in rodents than infection with *B. burgdorferi* but the proportion of the infected with *A. phagocytophilum* rodents did not correlate with the number of diagnosed patients. The most probable explanation of this fact is that anaplasmosis is unspecific febrile illness that can go unrecognized. However, our findings proved that the disease is much more common in Bulgaria than thought.

These results also revealed that *A. phagocytophilum* and *B. burgdorferi* differed in their preferences to rodent species. *A. agrarius* was found to be the preferable host for *A. phagocytophilum*, while *A. flavicollis* was the preferable host for *B. burgdorferi*.

In the USA, *Peromyscus* mice have been shown as the main reservoir host for *A. phagocytophilum* (Magnarelli *et al.* 1997, Telford *et al.* 1996, Walls *et al.* 1997). The equivalent role of *Apodemus* mice in Europe has been suspected (Liz *et al.* 2000, Ogden *et al.* 1998). Our finding that one third of *A. agrarius* were naturally infected with the agent of human anaplasmosis suggested that this rodent species most probably serve as a natural reservoir of the pathogen.

Many mammal species have now been determined as competent reservoirs of *B. burgdorferi* in Europe. The most important probably are mice (*Apodemus* spp.) and voles (*Clethrionomys* spp.) (Aeschlimann *et al.* 1986, Hu *et al.*1991, Humair *et al.* 1999, Matuschka *et al.* 1994, Randolf, Craine 1995, Tälleklint, Jeanson 1994, Zore *et al.* 1999). Our results also confirmed high rate if infection in *Apodemus* mice. These results also showed *Apodemus* mice as important reservoirs of *B. burgdorferi* in nature. These mice being preferable hosts for ticks are capable to disseminate widely the infection in various host species.

In conclusion, the prevalence of infection with *A. phagocytophilum* and *B. burgdorferi* in rodents elucidated their role in maintaining pathogens in the environment. The high frequency of the infection with both pathogens is an indicator for the risk of infection in humans. Further investigations are needed to detect possible co-infections and to analyze interactions between these pathogens.

Acknowledgements: This study was supported by Grant DDBU 02/26/20.12.2010 from the Ministry of Education, Youth and Science, Sofia, Bulgaria under Program for stimulating of scientific investigations in governmental high schools.

References

Aeschlimann A ., E. Chamot, F. Gigon, J.-P. Jeanneret, D. Kesseler and C. Walther 1986. *B. burgdorferi* in Switzerland. – *Zbl. Bakl. Hyg. A*, **262**: 450-458.

Hu C., P. Humaire P., R. Wallich, and L. Gern 1991. *Apodemus* sp. Rodents, reservoir hosts for *Borrelia afzelii* in an endemic area in Switzerland. – *Zbl. Bakl.*, **285**: 558-564.

Humair P., O. Rais and L. Gern 1999. Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes* ticks : differential transmission pattern and overwintering maintenance. – *Parasitology*, **118**: 33-42.

Liz J., L. Anderes, J. Sumner, R Massung, L. Gern,B. Rutti and M. Brossard 2000. PCR detection of granulocytic ehrlichiae in *Ixodes ricinus* ticks and wild small mammals in western Sitzerland. – *J. Clin. Microbiol.*, **38**: 1002-1007.

Magnarelli L., J. Anderson, K. Stafford III and J. Dumler 1997. Antibodies to multiple tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in white-footed mice. – *J. Wildl. Dis.*, **33**: 466-473.

Matuschka F.-R., P. Fischer, M. Heiler, D. Richter and A.Spielman 1994. Capacity of European animals as reservoir hosts for the Lyme disease spirochete. – *J. Infect. Dis.*, **165**: 479-483.

Matuschka F.-R., A. Endepolis, D Richter and A. Spielman 1997. Competence of urban rats as reservoir hosts for Lyme disease spirochetes. – *J. Med. Entomol.*, **34**: 489-493.

Mitchell P., K. Reed and J. Hofkes 1996. Immunoserologic evidence of coinfection with *Borrelia burgdorferi*, *Babesia microti*, and human granulocytic *Ehrlichia* species in residents of Wisconsin and Minnesota. – *J. Clin. Microbiol.*, **34**: 724-727.

Ogden N., K. Bown, B. Horrocks, Z. Woldehiwet and M. Bennett 1998. Granulocytic *Ehrlichia* infection in ixodid ticks and mammals in woodlands and uplands of the UK. – *Med. Vet. Entomol.*, **12**: 423-429.

Randolf S., N. Craine 1995. General framework for comparative quantitative studies on transmission of tick-borne diseases using Lyme borreliosis in Europe as an example. – *J. Med. Entomol.*, **2**: 765-777.

Schmidt B., R. Muellegger, C. Stockenhuber, H. Sover,S. Hoedl, A. Luger and H. Kerl 1996. Detection of *Borrelia burgdorferi*-specific DNA in urine specimens from patients with erythema migrans before and after antibiotic therapy. – *J. Clin. Microbiol*., **34**, 1359-63.

Tälleklint L., T. Jeanson 1994. Transmission of *Borrelia burgdorferi* s.l. from mammal reservoirs to the primary vector of Lyme borreliosis *Ixodes ricinus* (Acari: Ixodidae), in Sweden. – *J. Med. Entomol.*, **31**: 880-886.

Telford S. III, J. Dawson, P. Katavolos, C. Warner, C. Kolbert and D. Persing 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. – *Proc. Natl. Acad. Sci. USA*, **93**: 6209-6214.

Walls J., B. Greig, D. Neitzel and J. Dumler 1997. Natural infection of small mammal species in Minnesota with the agent of human granulocytic ehrlichiosis. – *J. Clin. Microbiol.*, **35**: 853-855.

Walls J., P. Caturegli, J. Bakken, K. Asanovich and J. Dumler 2000. Improved sensitivity of PCR for diagnosis of human granulocytic ehrlichiosis using *epank1* genes of *Ehrlichia phagocytophila* – group Ehrlichiae. – *J Clin Microbiol*, **38**: 354-356.

Zore A., M. Petrovec, K. Prosenc, T. Trilar, E. Ruzic-Sabljic and T. Avsic-Zupanc 1999. Infection of small mammals with *Borrelia burgdorferi* sensu lato in Slovenia as determined by polymerase chain reaction (PCR). – *Wien Klin. Wochenschr.*, **11**: 997-999.