

HARD TICKS (ACARI: IXODIDAE)-VECTORS FOR LYME DISEASE SPIROCHETES IN ROMANIA

ELENA CLAUDIA COIPAN

In this study 1868 questing *Ixodes ricinus* ticks (nymphs and adults), collected in six different sites from three counties (Giurgiu, Sibiu and Tulcea) in Romania, were examined by polymerase chain reaction (PCR), followed by reverse line blot (RLB) for detection of *Borrelia burgdorferi* sensu lato presence. The bacteria were found in 18.04 % of the investigated ticks. The prevalence of infection was higher in *Ixodes ricinus* nymphs (19.1 %) than in adults (15.37 %). Three *B. burgdorferi* sensu lato genospecies were detected: *B. afzelii* (61.13 %), *B. garinii* (31.16 %) and *B. valaisiana* (7.72 %). No mixed infections were detected in the investigated ticks. The highest infection prevalence in *I. ricinus* nymphs was detected at Cristian (Sibiu County) – 22.03 %, while in adults it was at Comana (Giurgiu County) – 19.77 %. This preliminary study provides evidence that Lyme disease spirochetes are present in different regions of Romania and at a relatively high prevalence in *Ixodes ricinus* vector, thus posing a risk of infection to human subjects that undergo work or leisure activities in the areas infested by ticks.

Key words: *Borrelia burgdorferi* sensu lato, *Ixodes ricinus*, ticks, Lyme disease, PCR-RLB, Romania.

INTRODUCTION

Romanian fauna of hard ticks (Acari: Ixodidae) comprises 25 species (Feider, 1965; Bădescu, 1967; Horak *et al.*, 2002; Kolonin, 2009). Among them, *Ixodes ricinus* L. is the most common and widespread species in Romania (Feider, 1965).

Many of these tick species are important vectors for different pathogens of both medical and veterinary importance. Ticks and the diseases they transmit have a zoogeographical range restricted by host movement and, to some extent, climatic factors. The increased mobility of pets has resulted in rapid extension of the zoogeographical ranges for many species (Shaw *et al.*, 2001). The zoogeographical range is also increasing because tick species are finding niches in different climatic conditions (Lindgren & Gustafson, 2001). Anthropogenic activities such as habitat fragmentation and export of wild animals for different purposes could determine the extension of ticks' zoogeographical ranges and influence wildlife pathogen outbreaks (Dobson & Foufopoulos, 2001). Thus, the dispersal of ticks could have significant impact in terms of economic (livestock and wildlife losses) and health aspects (modifications in the epidemiology of tick-borne diseases).

Ixodes ricinus L. is the main vector of *Borrelia burgdorferi* sensu lato, the etiological agents of Lyme borreliosis, in Europe (Eisen & Lane, 2002). Seven *Borrelia* genospecies have been found associated with this tick species and the risk of human infection with *Borrelia* depends on outdoor activity, on the density of tick populations, and on the infection of the ticks with *Borrelia*. Therefore, data describing the prevalence of *Borrelia* in ticks can be used to assess the risk of Lyme borreliosis for public health (Rauter & Hartung, 2005).

The aim of this study was to investigate the presence of *B. burgdorferi* s.l. in *Ixodes ricinus* ticks from different regions of the country.

MATERIAL AND METHODS

Study Areas and Tick Collection. The study was carried out in one site from Giurgiu County – Comana (N 44° 09076', E 26° 06589'), three sites from Sibiu County: Brateiu (N 46° 10100', E 024° 23825'), Cristian (N 45° 46783', E 023° 57782'), and Sadu (N 45° 38696', E 024° 08772'), and two sites from Tulcea County: Ciucurova (N 44° 53352', E 28° 29927') and Măcin (N 45° 09599', E 28° 18450') (Fig. 1). *Ixodes ricinus* ticks were collected in wooded areas (mixed forests) until 14.00 hours of the day. Host-seeking adult, nymphal, and larval ticks were collected monthly from March to October in two consecutive years – 2007 and 2008 – by flagging the low vegetation with a 1 m² white flag over a distance of 100 m. Collected ticks were kept in plastic tubes until they were identified at species level by examination under stereomicroscope, following the keys described by Feider (1965), Baker (1999) and Estrada-Peña *et al.* (2004). After that, they were passed into cryotubes containing RNAlater® (Ambion®, Applied Biosystems) solution and stored at –80 °C to the moment of examination for *Borrelia burgdorferi* s.l. infection.

Detection of *Borrelia burgdorferi* sensu lato in ticks. A number of 1341 nymphs and 527 adults of *Ixodes ricinus* were examined for *B. burgdorferi* s.l. genospecies by polymerase chain reaction (PCR), followed by the reverse line blot (RLB) assay.

DNA extraction was achieved by placing the tick in 100 µl 0.7 M ammonium hydroxide and boiling it for 15 minutes at 100°C (Guy & Stanek 1991; Rijpkema *et al.*, 1995).

PCR. Primers used to amplify the variable spacer region between two repeated genes encoding for ribosomal 23S and 5S were B5S-Bor and 23S-Bor primers (Schouls *et al.*, 1999; Alekseev *et al.*, 2001) and DNA amplification was performed in a 2720 Thermal Cycler (Applied Biosystems), following a touchdown PCR program previously described by Morán-Cadenas *et al.* (2007).

Agarose gel electrophoresis. PCR products were stained with ethidium bromide and visualized on a UV transilluminator (UVP), after agarose gel

electrophoresis (2% agarose, 1xTAE, pH 8.0), at 70V, 45 min, and stored at 4°C until RLB analysis. All samples that produced bands between 400 and 500 bp were subjected to DNA-DNA hybridization by the reverse line blot method.

Reverse Line Blotting. Isolates of *B. burgdorferi* sensu stricto (B31), *B. garinii* (NE11), and *B. afzelii* (NE632) (obtained by the amiability of Prof. Dr. Lise Gern, Institut of Zoology, University of Neuchâtel, Switzerland) were used as positive controls. Two negative controls were also used: one for DNA extraction and the other for PCR. The RLB technique was performed as described by Schouls *et al.* (1999) and Alekseev *et al.* (2001) using 5 different oligonucleotide probes (75 pmol and 100 pmol): *B. burgdorferi* s.l. (SL), *B. burgdorferi* sensu stricto (SS), *B. afzelii* (AF), *B. garinii* (GA), *B. valaisiana* (VS) (Rijkema *et al.*, 1995; Schouls *et al.*, 1999; Alekseev *et al.*, 2001). All probes were blotted in lines on an EDAC (*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, Sigma-Aldrich) activated Biotodyne C membrane (Pall Europe Ltd.) using a Miniblotter 45 (Immunetic). Hybridization was visualized by incubating the membrane with enhanced chemiluminescence detection liquid (GE Healthcare Europe) and by exposing the membrane to Amersham Hyperfilm ECL (GE Healthcare Europe).

Statistical analysis. Chi square and Fisher's exact test were calculated by using PASW® Statistics 17.0 (IBM SPSS®).

RESULTS

From the 1868 *Ixodes ricinus* ticks (1341 nymphs and 527 adults) analyzed for *B. burgdorferi* infection the target region was successfully amplified for ~ 18.04% of them. The number of infected nymphs was higher (19.1 %) than that of infected adults (15.37%) but not significantly (χ^2 test, $p = 0.0697$). Also, the number of infected ticks was slightly higher in 2007 (18.47 %) than in 2008 (17.78 %).

Regarding the monthly distribution of *B. burgdorferi* s.l. infection in *Ixodes ricinus* ticks a higher infection prevalence was noticed in May (27.21 % in 2007 and 27.33 % in 2008) and September (16.46 %) – October (19.45 %), in 2007 and 2008 respectively. Statistical analysis on the annual means of infection prevalence both within (between nymphs and adults) and between the sites, did not reveal any significant differences (χ^2 and Fisher tests with $p > 0.05$).

The highest infection prevalence in 2007 was observed at Cristian, for both stages (20 % in adults and 24 % in nymphs), while the lowest was observed at Măcin (5.26 % in adults and 12.9 % in nymphs). In 2008 similar infection prevalence patterns in nymphs were observed, meaning that the highest infection prevalence was again recorded at Cristian (21.03%) and the lowest at Măcin (8.33%). That was not the case for adults, where the highest infection prevalence was noticed at Comana (20 %) and the lowest at Sadu (5.88 %). The highest mean

(between the two years) infection prevalence in nymphs was detected at Cristian (Sibiu County) – 22.03 %, while in nymphs it was at Comana (Giurgiu County) – 19.77%.

The PCR products hybridized to the membrane on the rows corresponding to the probes for *Borrelia garinii*, *Borrelia afzelii* and *Borrelia valaisiana*. So, three *Borrelia* species were identified by PCR-RLB: *B. afzelii* was the most frequently detected species (61.13 %), with *B. garinii* (31.16 %) less common and *B. valaisiana* (7.72 %) quite rarely encountered and even missing from some of the sites (Sadu) (Fig. 1). *B. burgdorferi* sensu stricto was not detected in any of the sites. The ratio of spirochetes genospecies infecting *I. ricinus* ticks varied greatly between the different instars and between the sites (χ^2 p > 0.05). Thus, *B. afzelii* was detected in 90.91 % of the infected ticks at Sadu and in 40.3 % of them at Ciucurova; *B. garinii* varied from 47.76 % at Ciucurova to 9.09 % at Sadu, while for *B. valaisiana*, the maximum infection prevalence was found at Ciucurova (11.94 %) (Fig. 1).

The interannual and interstadial variation in the ratio of the genospecies infecting ticks were very low, statistically insignificant (interannual – $\chi^2 = 0.23$, p = 0.89, interstadial – $\chi^2 = 0.63$, p = 0.73).

DISCUSSION

This study confirmed the presence of *Borrelia burgdorferi* s.l. in $\approx 18\%$ of the questing *Ixodes ricinus* ticks collected in different regions of Romania. This prevalence is comparable to the overall prevalence average in Europe which ranges from 0 to 47% (Gray *et al.*, 1998; Jouda *et al.*, 2004; Rauter & Hartung, 2005) and is close to the ones reported in countries like Slovenia (19% – Strle *et al.*, 1995) or Austria (23.2% – Hubalek *et al.*, 2003) where human Lyme borreliosis incidence reaches its utmost values for the Old World – 155 and 130, respectively, new cases for every 100,000 inhabitants (Lindgren & Jaenson, 2006; EU-CALB, 2010).

Both nymphal and adult *Ixodes ricinus* were proven to be infected with the bacteria. The lower infection prevalence in adults than in nymphs indicates that a large percentage of nymphs feed on host species non-competent to transmit *B. burgdorferi* s.l., most likely deer (Jaenson & Tälleklint, 1992; Randolph & Craine, 1995; Gray *et al.*, 1999). Furthermore, the results confirm the assertion that *B. afzelii* is the dominant *B. burgdorferi* s.l. genospecies followed by *B. garinii* (Rauter & Hartung, 2005). The fact that *B. burgdorferi* s.s., which across Europe is almost equally prevalent with *B. valaisiana*, was not encountered may find an explanation in that its frequency in Europe seems to decrease from west to east (Saint Girons *et al.*, 1998).

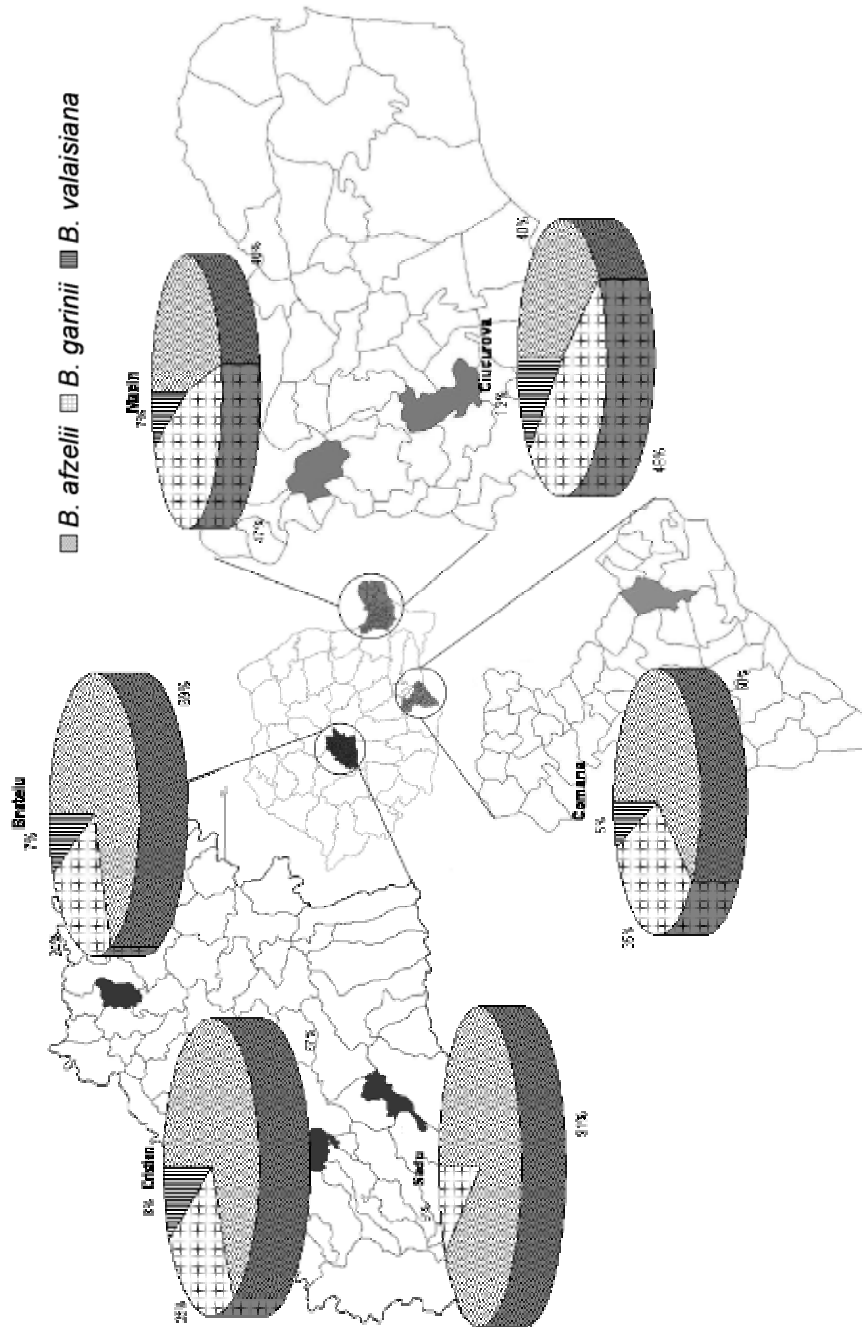


Fig. 1 . Ratio of *B. burgdorferi* s.l. genospecies detected in *I. ricinus* in different sites.

The wide variations between sites in the proportion of infecting *Borrelia* genospecies could be due to some differences in available vertebrate host assemblages and particularly those with reservoir competence for the spirochetes. It is well known the fact that *B. burgdorferi* s.l. genospecies show vertebrate host specificity, independently of the extrinsic ecological factors (Kurtenbach *et al.*, 1998; Hu *et al.*, 2001). Accordingly, the proportion of different *B. burgdorferi* s.l. genospecies in a *site* is a function of the number and density of the reservoir-competent species in that site. Thus, the high prevalence of *B. afzelii* (61.13 % of the positive *Ixodes ricinus* ticks) comparing to *B. garinii* (31.16 %) may be related to the enzootic cycles that are predominant in the investigated area. For example, in Sibiu County there is an abundant rodent fauna, with *Apodemus flavicollis* and *A. agrarius* as the dominant species (Benedek, 2008), and a high percentage of *Ixodes ricinus* ticks infected with *B. afzelii* is consistent with the known host specificity of ticks and spirochetes (Matuschka *et al.*, 1991).

Most probably the interannual variation of genospecies ratio does not have any biological significance and is the sheer result of hazard in collecting the ticks that fed on different host species. The similar patterns of infecting genospecies in both investigated tick stages may be proof of ticks' equal chances of feeding on reservoir-competent hosts during their life-cycle.

CONCLUSIONS

Ixodes ricinus (Acari: Ixodidae) is the most common and widespread tick species in Romania. This is the first study to assess *Borrelia burgdorferi* s.l. infection prevalence in *Ixodes ricinus* ticks from different regions of Romania (six sites from three counties) using polymerase chain reaction (PCR), followed by the reverse line blot (RLB) assay. In 1868 *Ixodes ricinus* ticks (1341 nymphs and 527 adults) analyzed for *B. burgdorferi* s.l. infection three genospecies were detected: *B. afzelii*, *B. garinii* and *B. valaisiana*, at an overall infection prevalence of 18.04%, with maximum values detected at Cristian (Sibiu County) and Comana (Giurgiu County) for *Ixodes ricinus* nymphs and adults respectively.

Interannual variation in the infection prevalence was assessed, indicating comparable infection rates in the two consecutive years of study. Infection prevalence and genospecies ratio varied greatly between sites, even from the same region, indicating that different host and vegetation assemblages lead to different combinations of circulating spirochetes.

The detection of only three *Borrelia* genospecies probably does not reflect the reality from the field, and a larger number of investigated ticks, covering more regions and habitats would be necessary for an ensemble view of *B. burgdorferi* s.l. genospecies circulating in Romania.

This preliminary study provides evidence that Lyme disease spirochetes are present in different regions of Romania, and at a relatively high prevalence in

Ixodes ricinus vectors, thus posing a risk of infection to humans that undergo work or leisure activities in the areas infested by ticks.

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REFERENCES

- ALEKSEEV A.N., DUBININA H.V., VAN DE POL I., SCHOOLS L.M., 2001, *Identification of Ehrlichia spp. and Borrelia burgdorferi in Ixodes ticks in the Baltic regions of Russia*. Journal of Clinical Microbiology, **39**: 2237-2242.
- BAKER A.S., 1999, *Mites and ticks of domestic animals, An identification guide and information source*. The Stationery Office, London, 240 pp.
- BĂDESCU C., 1967, *Ixodes arboricola Schulze și Schlotke 1929, o nouă specie a subgenului Pholeoixodes Sch. 1942 din Republica Socialistă România*. Lucrări Științifice, Seria C, Zootehnie și Medicină Veterinară, **10**: 329-338.
- BENEDEK A.M., 2008, *Studii asupra mamiferelor mici (Ordinele Insectivora și Rodentia) în Transilvania-România*. PhD Thesis, University of Bucharest, Romania.
- DOBSON A.P., FOUFOPOULOS J., 2001, *Emerging infectious pathogens in wildlife*. Philosophical Transactions of the Royal Society of London B, **356**: 1001-1012.
- EISEN L., LANE R.S., 2002, *Vectors of Borrelia burgdorferi sensu lato*. Pp.: 91-115. In: Gray J., Kahl O., Lane R.S. and Stanek G. (Eds.), *Lyme borreliosis: Biology, Epidemiology and Control*, CABI Publishing, Wallingford, Oxfordshire.
- ESTRADA-PEÑA A., BOUATTOUR A., CAMICAS J.-L., WALKER A.R., 2004, *Ticks of domestic animals in the Mediterranean region*. Ed. ICTTD, Zaragoza, 131 pp.
- FEIDER Z., 1965, *Arachnida Acaromorpha, Suprafamilia Ixodoidea (Căpușe)*. Pp.: 89-104. In: *Fauna R.P.R.*, Vol. V, Fasc. 2, Edit. Acad. R.P.R.
- GRAY J.S., KAHL O., ROBERTSON J.N., DANIEL M., ESTRADA-PEÑA A., GETTINBY G., JAENSON T.G., JENSEN P., JONGEJAN F., KORENBERG E., KURTENBACH K., ZEMAN P., 1998, *Lyme borreliosis habitat assessment*. Zentralblatt für Bakteriologie, **287**: 211-228.
- GRAY J.S., KIRSTEIN F., ROBERTSON J.N., STEIN J., KAHL O., 1999, *Borrelia burgdorferi sensu lato in Ixodes ricinus ticks and rodents in a recreational park in South-Western Ireland*. Experimental and Applied Acarology, **23**: 717-729.
- GUY E.C., STANEK G., 1991, *Detection of Borrelia burgdorferi in patients with Lyme disease by the polymerase chain reaction*. Journal of Clinical Pathology, **44**: 610-611.
- HORAK I.G., CAMICAS J.L., KEIRANS J.E., 2002, *The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida): a world list of valid tick names*. Experimental and Applied Acarology, **28**: 27-54.
- HU C.M., WILSKE B., FINGERLE V., LOBET Y., GERN L., 2001, *Transmission of Borrelia garinii OspA serotype 4 to BALB/c mice by Ixodes ricinus ticks collected in the field*. Journal of Clinical Microbiology, **39**: 1169-1171.
- HUBALEK Z., STUNZNER D., HALOUZKA J., SIXL W., WENDELIN I., JURICOVA Z., SANOGO Y.O., 2003, *Prevalence of borreliae in ixodid ticks from a floodplain forest ecosystem*. Wiener Klinische Wochenschrift, **115**: 121-124.
- JAENSON T.G., TÄLLEKLINT L., 1992, *Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete*. Journal of Medical Entomology, **29**: 813-817.

- JOUDA F., PERRET J.L., GERN L., 2004, *Ixodes ricinus* density, and distribution and prevalence of *Borrelia burgdorferi sensu lato* infection along an altitudinal gradient. *Journal of Medical Entomology*, **41**: 162-169.
- KOLONIN G.V., 2009, *Fauna of ixodid ticks of the world (Acari: Ixodidae)*. Moscow. on-line
- KURTENBACH K., PEACEY M., RIJKEMA S.G., HOODLESS A.N., NUTTALL P.A., RANDOLPH S.E., 1998, *Differential transmission of the genospecies of Borrelia burgdorferi sensu lato by game birds and small rodents in England*. *Applied and Environmental Microbiology*, **64**: 1169-1174.
- LINDGREN E., GUSTAFSON R., 2001, *Tick-borne encephalitis in Sweden and climate change*. *Lancet*, **358**: 16-18.
- LINDGREN E., JAENSON T.G.T., 2006, *Lyme borreliosis in Europe: influences of climate and climate change, epidemiology, ecology and adaptation measures*. Pp.:157-188. In: B. Menne & K.L. Ebi (Eds.), *Climate Change and Adaptation Strategies for Human Health*. Springer, Darmstadt & WHO, Geneva.
- MATUSCHKA F.-R., FISCHER P., MUSGRAVE K., RICHTER D., SPIELMAN A., 1991, *Hosts on which nymphal Ixodes fcinus most abundantly feed*. *American Journal of Tropical Medicine and Hygiene*, **44**: 100-107.
- MORÁN-CADENAS F., SCHNEIDER H., LOMMANO E., BURRI C., MORET J., GERN L., 2007, *A comparison of two DNA extraction approaches in the detection of Borrelia burgdorferi sensu lato from Ixodes ricinus ticks by PCR and Reverse Line Blotting*. *Vector Borne and Zoonotic Diseases*, **7**: 555-562.
- RANDOLPH S.E., CRAINE N.G., 1995, *General framework for comparative quantitative studies on transmission of tick-borne diseases using Lyme borreliosis in Europe as an example*. *Journal of Medical Entomology*, **32**:765-777.
- RAUTER C., HARTUNG T., 2005, *Prevalence of Borrelia burgdorferi sensu lato genospecies in Ixodes ricinus ticks in Europe: a metaanalysis*. *Applied and Environmental Microbiology*, **71**: 7203-7216.
- RIJKEMA S.G., MOLKENBOER M.J., SCHOOLS L.M., JONGEJAN F., SCHELLEKENS J.F., 1995, *Simultaneous detection and genotyping of three genomic groups of Borrelia burgdorferi sensu lato in Dutch Ixodes ricinus ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes*. *Journal of Clinical Microbiology*, **33**: 3091-3095.
- SAINT GIRONS I., GERN L., GRAY J.S., GUY E.C., KORENBERG E., NUTTALL P.A., RIJKEMA S.G., SCHONBERG A., STANEK G., POSTIC D., 1998, *Identification of Borrelia burgdorferi sensu lato species in Europe*. *Zentralblatt fur Bakteriologie*, **287**: 190-195.
- SCHOOLS L.M., VAN DE POL I., RIJKEMA S.G.T., SCHOT C.S., 1999, *Detection and identification of Ehrlichia, Borrelia burgdorferi sensu lato, and Bartonella species in Dutch Ixodes ricinus ticks*. *Journal of Clinical Microbiology*, **37**: 2215-2222.
- SHAW S.E., DAY M.J., BIRTLES R.J., BREITSCHWERDT E.B., 2001, *Tick-borne infectious diseases of dogs*. *Trends in Parasitology*, **17**: 74-80.
- STRLE F., CHENG Y., NELSON J.A., PICKEN M.M., BOUSEMAN J.K., PICKEN R.N., 1995, *Infection rate of Ixodes ricinus ticks with Borrelia afzelii, Borrelia garinii, and Borrelia burgdorferi sensu stricto in Slovenia*. *European Journal of Clinical Microbiology and Infectious Diseases*, **14**: 994-1001.
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“Cantacuzino” National Institute of Research-Development
for Microbiology and Immunology
103 Splaiul Independenței Street, 050096, Bucharest,
Romania
e-mail: elenaclaudiarv@yahoo.co.uk

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