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DISTRIBUTION AND ECOLOGICAL FEATURES OF BORRELIA BURGDORFERI SENSU LATO GENOSPECIES PREVALENT IN URBAN AND SYLVATIC BIOCENOSES FROM NORTHWEST TRANSYLVANIA

SUMMARY OF Ph.D. THESIS

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SUMMARY

Lyme borreliosis is caused by spirochetes from the *Borrelia burgdorferi* s.l. complex, transmitted by *Ixodes* ticks; it affects humans, but also domestic animals like dogs and horses, and it is considered the most widespread vector-borne disease of the northern hemisphere. Also called ”the disease with one thousand faces”, due to the extremely varied symptoms which can mimic a large number of conditions, Lyme borreliosis is a multisystemic disease that involves skin, joints, the heart and the nervous system, with an estimated number of 65.500 patients per year in Europe, between 1 and 350 cases to 100.000 inhabitants (Hubálek, 2009).

In some areas of Europe (Hubálek, 2009), and in Romania (CNSCBT, 2010, 2011), the number of cases follows an increasing trend, but the difference may be attributed to a heightened awareness of Lyme disease in medical practitioners and in general public alike, following the last years' intense mediatization of the risks implied by a tick bite. Vector ticks are spreading to higher altitudes and latitudes, suggesting that Lyme borreliosis will continue to represent a subject of interest for public health, especially in the light of economic and climatic predictions. The effect of reduced biodiversity and habitat changes on the emergence and persistence of Lyme disease foci has to represent an important subject of scientific research, especially for the identification of a new prevention and control paradigm of Lyme borreliosis and other tick-borne diseases. It is clear that a complete eco-epidemiological characterization and appropriate risk evaluation modeling is possible only through concerted efforts of specialists from various fields, such as medicine, veterinary medicine, epidemiology, parasitology, microbiology, immunology.

The studies described in this Ph.D. thesis have been conducted between 2008-2012, and followed the general purpose of revealing certain aspects regarding the presence, spread, transmission, diagnosis and prevention of *B. burgdorferi* s.l. infection, with a special reference to Cluj county or Transylvania region, according to the given situation. Because Lyme disease is a vector-borne zoonosis which affects mainly humans, the trait that the epidemiologic and ecologic aspects followed by this thesis have in common is their utility in risk evaluation of *B. burgdorferi* s.l. exposure in a given habitat, and of the factors that can influence this. Both epidemiologic and experimental studies have been performed, implicating every link of the spirochete transmission cycle. The objectives can be summarized as follows:

- establishing prevalence of *B. burgdorferi* s.l. in vector tick populations from urban and suburban habitats, both unfed and collected from hosts, represented in this case by northern white-breasted hedgehog (*Erinaceus roumanicus*);
- estimating average feeding time of *I. ricinus* ticks collected from 3 different host species represented by humans, dogs and horses, and based on these figures, evaluation of disease transmission risk;
- establishing seroprevalence of anti-*B. burgdorferi* s.l. antibodies in populations of dogs and horses from different counties of Transylvania;
- investigating the diagnostic and screening value of SNAP 4Dx® rapid test in dogs and evaluating its applicability in horses;
- testing the borrelicidal effect due to complement activity of horse serum;
- investigating the value of the wild boar (Sus scrofa) as a sentinel species, based on number of confirmed human cases;
- testing the borrelicidal effect due to complement activity of wild boar serum against different strains of B. burgdorferi s.l.

The thesis has 205 pages and it is structured in 2 main parts, the first one entitled "Literature review” which comprises 43 pages, and the second one named "Original research”, extended on 162 pages. It contains 32 figures (photo images, graphic representations, charts) and 29 tables.

The first part represents a synthesis of the scientific literature on Lyme disease, divided in 6 chapters. The first chapter is a review of the importance and history of Lyme disease. The second one describes the symptoms of the disease, in humans and in domestic animals, emphasizing the clinical differences between European and North American cases, generated by the various genospecies spread on each continent. The third chapter describes the morphology and structure of the pathogen, discussing in depth the characteristics that influence pathogenicity and antigenicity. The fourth chapter summarizes the different methods of demonstrating the presence of B. burgdorferi s.l. or the contact with the spirochete in various sample types. The fifth chapter gives an insight on the complex relationships between the pathogen, the vector and the host. The last chapter of the first part describes some aspects of Lyme borreliosis ecology and the particularities of epidemiologic cycles in Europe.

The second part of the thesis is aiming to present the results of original research. It begins with a summary of the objectives, and the presentation of the experimental parts is followed by general conclusions and the list of references, which includes 365 titles.

The studies included in chapters II.I.I. and II.I.II. were aimed at the vectors of Lyme borreliosis, ixodid ticks. The main vector in Europe is represented by I. ricinus ticks, the most widely spread tick species of this continent (Ekner et al., 2011). These ticks have a low host specificity, feeding on numerous species of mammals, birds and reptiles. Their life cycle comprises 3 parasitic developmental stages (larva, nymph, adult - female and male), every moult being preceded by a blood meal on a different host (Gern, 2008). With every feeding, the parasite has the chance of becoming infected with B. burgdorferi s.l., or transmitting the infection to a susceptible host.

Schauber și Ostfeld (2002) hypothesized that the number of ticks attached to small mammals tend to vary less compared to the number of ticks obtained by flagging, thus giving a better estimate of tick population density in a given area. Evaluating the prevalence of B. burgdorferi s.l. infection in I. ricinus tick populations and determining the diversity of genospecies involved can be regarded as an indicator of Lyme borreliosis risk for humans in a certain habitat (Cisak et al., 2006).

The main objective of chapter II.I.I. was to evaluate the prevalence of B. burgdorferi s.l. in general, and that of B. afzelii, B. garinii and B. burgdorferi s.s. in particular, both in
unfed ticks captured by flagging and collected from hosts, in this case from hedgehogs (*E. roumanicus*). The studied areas were represented by urban habitats, situated within the perimeters of Cluj-Napoca, or in its close proximity. Secondarily, a description of tick community composition of questing ticks, and a quantitative and qualitative characterization of tick parasitism in the northern white-breasted hedgehog (*E. roumanicus*) was aimed at.

In the frame of this study, 229 ticks were collected by flagging from 5 different areas located in the city of Cluj-Napoca or in the close proximity, and 451 ticks from 12 hedgehogs (*E. roumanicus*), captured in 3 of the areas mentioned above. The identification of developmental stage and species of the collected parasites was performed, and *I. ricinus* ticks were further tested for the presence of *B. burgdorferi* s.l. in their organism. This was carried out using primers that delineate a common region for all genospecies, in the setting of a classical PCR reaction. The identification of the 3 pathogenic genospecies followed was possible using RFLP (restriction fragment length polymorphism), coupled with PCR using genospecies-specific primers.

Results revealed that at least 4 tick species can be found in parks and recreational areas of Cluj-Napoca, the most abundant being *I. ricinus* (447/680, 65.73%), followed by *H. concinna*. This was true for every location except area E, a natural reserve, located in the close proximity of Cluj-Napoca, where the majority of ticks were represented by *D. marginatus*. Of the 4 identified species, *I. ricinus* and *H. concinna* were also found attached to hedgehogs. Data regarding tick community composition in the studied areas is detailed in fig. 1.

![Figure 1](image_url)

**Figure 1.** Graphic representation of the community structure found in tick echantions collected from the vegetation (a) and from hedgehogs (*E. roumanicus*) (b) in the studied areas.
The prevalence of tick infestation of *E. roumanicus* in the studied urban habitats was 75% (95% CI: 45.72% - 92.81%), with a mean intensity of 50.11 parasites per host (95% CI: 21.44 - 98.78).

Regarding the prevalence of *B. burgdorferi* s.l. infection in ticks, a value of 10.76% (7/65), and 14.65% (56/382) was calculated for unfed ticks, and ticks collected from hosts, respectively. The difference was not statistically significant. There was no statistical difference between locations, except area B, where the percent of positive ticks was significantly lower (p = 0.001). Conversely, the developmental stage had a significant effect on the rate of infection in both tick categories, the number of positive larvae being considerably lower compared to that of positive nymphs or adults (p ≤ 0.001). This is due to the fact that transovarial transmission of *B. burgdorferi* s.l. is extremely rare (Ginsberg, 2008).

All 3 followed pathogenic genospecies, namely *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s., were identified in positive *I. ricinus* ticks from urban habitats, 42.85% of infected questing ticks and 41.07% of infected ticks collected from hedgehogs harbingering at least one pathogenic genospecies in their organism. In 11.11% (7/63) of the infected ticks, the presence of 2 pathogenic genospecies was demonstrated, with all possible combinations being identified. In fact, the percent of coinfections is probably higher, if one considers the genospecies which remained unidentified in this study.

In conclusion, demonstrating the presence of the pathogen and its vectors in all of the studied habitats, represented by parks and recreational areas, confirms that these meet the criterias necessary for maintaining active epidemiological transmission cycles of *B. burgdorferi* s.l., and that the risk of contracting Lyme disease is present even in urban habitats.

With the majority of tick-borne diseases, including Lyme borreliosis (Schwan and Piesman, 2000), the pathogen has to undergo a process of adaptation that enables it to successfully pass from the tick organism to the vertebrate host, and this adaptation requires a certain amount of time. Therefore, a window of opportunity exists during which, if the parasite is removed, the infection cannot be accomplished.

The small size of immature stages make them very hard to detect with the naked eye, but even adult unfed individuals that attack humans are often missed in the first stages of feeding (Logar et al., 2002). In domestic animals, the presence of a hairy coat, the lack of efficient grooming behaviour capable of removing the parasites and, often, the ignorance of the owners with respect to the dangers of tick exposure, contribute to the frequent lack of detection, which allows the ticks to feed to repletion and succesfully transmit pathogens.

The main objective of chapter II.I.II. was to evaluate mean feeding time in *I. ricinus* ticks collected from 3 different host species, namely humans, dogs and horses, and, based on these figures, the estimation of the Lyme borreliosis transmission risk. As secondary objectives, the identification of ixodid tick species parasitising the 3 host species in Cluj county, and the evaluation of *B. burgdorferi* s.l. infection prevalence in ticks feeding on humans and domestic animals naturally followed.

The study included 185 ticks from 70 hosts belonging to 3 different species (43 dogs, 15 horses, 12 humans), collected by veterinarians, veterinary students or other individuals
from Cluj county. For estimation of feeding time, undamaged *I. ricinus* nymphs and females were selected, with a final number of 139 ticks being measured, of which 93 (91 females and 2 nymphs) were collected from dogs, 11 (females) from horses and 8 (4 females and 4 nymphs) from humans. Measurement of body parameters was performed according to the method described by Falco et al. (1996) and Gray et al. (2005); based on this data, scutal and coxal indexes were calculated, and afterwards, the feeding time was estimated for each *I. ricinus* tick individually.

The 185 examined ticks belonged to 4 species, the majority (160/185, 86.49%) being identified as *I. ricinus*. The other 3 species were present in much smaller numbers, with *D. marginatus* (21/185, 11.35%) being identified only in horses, *D. reticulatus* (2/185, 1.08%) only on dogs, and *H. concinna* (2/185, 1.08%) on humans and dogs. In horses, the most abundant tick species was *D. marginatus*. A significantly higher number of nymphs (p < 0.001) were found on humans, compared to ticks from domestic animals, which were almost exclusively represented by adults.

Mean feeding time of ticks differed significantly with the host species from which they were collected from (Kruskal-Wallis test, $\chi^2 = 155.35$; p < 0.001), the feeding time recorded in ticks attacking humans being significantly lower compared to those attached to dogs and horses (Fig. 2)

![Figure 2](image-url)  

**Figure 2.** Boxplot representation of feeding times recorded in *I. ricinus* ticks collected from 3 host species
Acknowledging the fact that *Ixodes ricinus* can transmit *B. burgdorferi* s.l. (especially *B. afzelii*) in under 17 hours of attachment (Kahl et al., 1998), the ticks were assigned to 3 risk groups based on the value of their feeding time (Table 1).

**Table 1.** Distribution of *I. ricinus* ticks in 3 risk categories according to estimated feeding time

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Dog</th>
<th>Horse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (&lt; 16 h)</td>
<td>9</td>
<td>0</td>
<td>6*</td>
</tr>
<tr>
<td>Moderate (16.1 - 48 h)</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>High (&gt; 48.1 h)</td>
<td>75</td>
<td>11</td>
<td>1*</td>
</tr>
</tbody>
</table>

Statistically significant differences (* - p < 0.001) were observed between the number of ticks collected from humans, included in the low and high risk groups, and the number of ticks collected from animals, included in the same categories.

From a total of 160 *I. ricinus* ticks, infection with *B. burgdorferi* s.l. was confirmed in 15 adult individuals (9.37%), in 3 of these one of the followed pathogenic genospecies was also identified (2 *B. afzelii*, 1 *B. garinii*).

Early removal of ticks is considered to be one of the most efficient measures for the prevention of Lyme borreliosis. The results of this study indicate that *I. ricinus* ticks, even immature stages, are observed and removed relatively fast in humans. Their significantly longer attachment time in dogs and horses is translated in an increased risk of successful transmission of *B. burgdorferi* s.l. spirochetes in these species, which warrants the use of rapidly acting acaricidal substances for the protection of these animals against *B. burgdorferi* and other tick-borne pathogens.

**Chapters II.III.** and **II.III.** deal with aspects regarding the epidemiology and diagnosis of Lyme borreliosis in dog populations from Transylvania.

Only about 5% of dogs infected with *B. burgdorferi* s.l. show clinical signs (Leschnik et al., 2010), most frequently involving the locomotor system, and a diagnosis of Lyme borreliosis is not easy to establish. In human medicine, two-tiered testing is a widely accepted serological diagnosis standard. It involves a preliminary testing using screening methods (ELISA or IFA), with the positive and borderline results being retested for confirmation by Western Blot (Wilske et al., 2007).

Despite the relatively low clinical importance of Lyme disease in canine pathology, dogs play an important role in the eco-epidemiological evaluation of its spreading. This species can be used as sentinels, and data regarding the exposure of dogs to *B. burgdorferi* s.l. can offer valuable information on the potential for human infection in a certain area (Duncan et al., 2005).
The main objective of chapter II.II.I. consisted in establishing seroprevalence of anti-*B. burgdorferi* s.l. antibodies in dog populations from urban and rural areas, along with the evaluation of the effect that several factors may have on this value.

The study comprised 298 dogs from 7 different counties in Transylvania; for each animal, data regarding age, gender and occupation was recorded. Based on this information, dogs were divided in several categories. Thus, for gender, 2 categories were created (male and female), for age, 3 categories (young animals: 0-2 years, adults: 3-7 years, old animals: over 8 years), and for occupation, 3 categories (pet, working dogs, including guard dogs, hunting dogs, shepherd dogs, and stray). Serological testing was performed using Lyme Borrelia Canine IgG - ELISA and Borrelia burgdorferi Lyme Blot Canine IgG - Western Blot (NovaTec Immunodiagnostica, GmbH, Germany) commercial kits.

In the studied dog populations, a total seroprevalence of 6.04% (18/298) was established, with the highest values recorded in Bihor and Hunedoara counties (25%), and the lowest ones in Alba and Maramureș counties (0%). Detailed results regarding county of origin and putative risk factors are presented in table 2.

Table 2. Distribution of seroreactive dogs according to county of origin, gender, age group and occupation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>No. of positive animals/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>County</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alba</td>
<td></td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>Bihor</td>
<td></td>
<td>3/12 (25) *</td>
</tr>
<tr>
<td>Brașov</td>
<td></td>
<td>6/28 (21.42) *</td>
</tr>
<tr>
<td>Cluj</td>
<td></td>
<td>5/180 (2.77)</td>
</tr>
<tr>
<td>Hunedoara</td>
<td></td>
<td>3/12 (25) *</td>
</tr>
<tr>
<td>Maramureș</td>
<td></td>
<td>0/45 (0)</td>
</tr>
<tr>
<td>Mureș</td>
<td></td>
<td>1/10 (10)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>8/171 (4.67)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>10/127 (7.87)</td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td></td>
<td>1/65 (1.53)</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td>15/196 (7.65)</td>
</tr>
<tr>
<td>Old</td>
<td></td>
<td>2/37 (5.40)</td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pet</td>
<td></td>
<td>3/99 (3.03)</td>
</tr>
<tr>
<td>Working</td>
<td></td>
<td>13/124 (10.48) **</td>
</tr>
<tr>
<td>Stray</td>
<td></td>
<td>2/85 (2.35)</td>
</tr>
</tbody>
</table>

Seropositivity recorded in Bihor, Brașov and Hunedoara counties was significantly higher (*, p ≤ 0.001) compared to the other counties. Regarding the samples from Brașov county, all the 6 positive results originated from one single locality, the village Arini, the seroprevalence recorded here being significantly higher compared to other settlements from the same county (p = 0.004).

Working dogs had a significantly higher percent of reactive individuals compared to pet or stray dogs (**, p = 0.025), the more frequent exposure of this category to an infected
tick bite being a plausible explanation of this fact. No significant influence of age and sex on seropositivity was recorded. The limited persistence of anti-*B. burgdorferi* s.l. antibodies does not allow the detection of immune responses older than 2 years, which excludes an increase of infection rate over time due to the accumulation of positive animals.

In conclusion, dogs from various parts of Transylvania are exposed to Lyme borreliosis spirochete, especially hunting, guard and shepherd dogs. Also, the results suggest a marked focal spreading of the pathogen, both on a large scale (among various counties), and on a smaller one (inside a particular county).

When faced with a dog showing clinical signs suggestive of Lyme borreliosis, the veterinarian often uses serological methods to confirm the condition, the "gold standard" being represented by two-tiered testing. A more convenient alternative is represented by rapid tests such as SNAP 4Dx® (Idexx Laboratories, SUA), which offers diagnosis for 4 vector-borne diseases of dogs, and results are obtained in less than 15 minutes. In case of borreliosis, the test detects antibodies directed against a synthetic peptide named C6, homologous of the invariant region IR6 of the surface protein VlsE (Cardoso et al., 2012).

The aim of chapter II.II.II. was to evaluate the diagnostic value of SNAP 4Dx® rapid test in identifying anti-*B. burgdorferi* s.l. antibodies in dogs, by comparing the results with those obtained from the two-tiered testing. 25 serum samples were included, priorly tested using ELISA and Western Blot. Of these, 18 samples were positive as confirmed by Western Blot, 3 samples reacted either positively or were in the grey zone after the ELISA testing, but were not confirmed by Western Blot, and 4 samples were negative after the ELISA testing.

The SNAP 4Dx® test identified correctly 8 out of the 18 positive samples, as well as all the 7 negative samples. For the remaining 10 samples, which were positive after ELISA and Western Blot, the rapid test showed a false negative result. No false positive results were recorded. The intrinsic diagnostic parameters of the test were: sensitivity - 44% (95% CI: 21.5-69.2%); specificity - 100% (95% CI: 59-100%); ROC curve analysis (Receiver Operating Characteristic - fig. 3) revealed an AUC (area under ROC curve) value of 0.722 (95% CI: 0.508 - 0.881), which corresponds to a low diagnostic value according to Zhu et al. (2010). It has to be pointed out that diagnostic value of a test in a certain population depends a lot on the prevalence of the disease in that population. This is why the calculations for positive and negative predictive values and accuracy were performed according to the Bayesian theory (Farr and Shapiro, 2000), which takes prevalence into consideration, in this case, 6.04%. A positive predictive value of 100% (95% CI: 50-100%), a negative predictive value of 96.5% (95% CI: 80-100%) and an accuracy of 96.6% were obtained.
Gottner et al. (2004) showed that differences in aminoacid sequences of IR6 region of various *B. burgdorferi* s.l. genospecies lead to differences in antigenicity, which in turn may lead to different seroreactivity of pacientes, according to the genospecies to which they have been exposed. The diversity of *B. burgdorferi* s.l. genospecies circulating in Europe may explain the low sensitivity recorded. Another possible explanation may be that anti-C6 antibodies dissapear after succesful treatment, a positive result being an indicator of active infection (Philipp et al., 2001; Philipp et al., 2005; Krupka et al., 2009); this aspect is particularly useful in monitoring treatment success, but in a serological survey would not permit the identification of animals exposed to the pathogen, but whose immune system prevented the infection.

In conclusion, a positive result of the SNAP 4Dx® rapid test is useful in a clinical setting, indicating an active infection with *B. burgdorferi* s.l., but a negative result must be interpreted cautiously. As a screening test, SNAP 4Dx® can be used with good results in the presence of a low seroprevalence.

Besides dogs, horses represent the domestic species in which Lyme borreliosis has been the most intensively studied, even if the percent of animals that develop clinical signs after an infection is low. The classical serological confirmation method implies a two-tiered testing, after the model described for human diagnosis, but Johnson et al. (2008) validated the use of SNAP 4Dx® rapid test for detection of anti-*B. burgdorferi* s.l. antibodies in horses, reporting a sensitivity of 63% and a specificity of 100%. Even if Lyme disease appears to have a rather small contribution to equine pathology, even in endemic foci, serologic surveillance of equine populations can be useful for evaluating the epidemiologic situation of a certain area, the role of this species as sentinels being unerlined previously by Maurizi et al. (2010) and Hansen et al. (2010).

The objective of **chapter II.III.I.** was to establish seroprevalence of anti-*B. burgdorferi* s.l. antibodies in horse populations from 5 counties in Transylvania. The
The purpose of retesting positive samples with SNAP 4Dx® rapid test was to compare the frequency of immune response to whole cell antigens of *B. burgdorferi* s.l. to that elicited against C6 synthetic antigen, in view of the fact that the latter is considered to be a marker of active infection.

A total number of 277 serum or plasma samples collected from horses in 5 different counties were tested for the presence of anti-*B. burgdorferi* s.l. antibodies. The horses were kept in different environments, both leisure and draft horses being included. To the extent it was possible, for each animal data was recorded regarding age, gender and occupation. Based on this information, the horses were divided in several categories. Thus, for gender, 2 categories were established (male and female), for age, 3 categories (young animals: 0-5 years, adults: 6-15 years, old animals: >16 years), and for occupation, 2 categories (leisure and draft). Serological testing was performed using MegaScreen® FluoBorrelia horse (MegaCor Diagnosik, Austria) commercial IFA kit, according to the manufacturer's instructions. Positive samples were retested using SNAP 4Dx® (Idexx Laboratories, SUA).

A global seroprevalence of 13.71% (38/277) was established in the studied horse populations. The county with the highest number of reactive horses was Maramureș, with a value of 30% (6/20), while the lowest value, 10.71% (3/28) was recorded in Satu Mare county, without this difference being statistically significant. Also, no statistically significant effect of gender, age or occupation on seroprevalence was identified. Of the 38 samples with antibody titers against *B. burgdorferi* s.l. detectable with IFA, 14 samples (36.84%) reacted positively to SNAP 4Dx® testing; significant differences between seroprevalence in different gender, age or occupation groups were not observed in this case either.

Metcalf et al. (2008) obtained positive results with the rapid test in only 2 out of 29 infected horses, for the animals with the highest titres of anti-*B. burgdorferi* s.l. antibodies. The correct identification of the individuals with the highest titres, that is the same animals more likely to develop clinical signs of Lyme disease (Manion et al., 1998), suggests that in a clinical setting the SNAP 4Dx® test could be useful, especially in the case of a positive result, but the interpretation of negative results should be done cautiously. The choice of either IFA or SNAP 4Dx® testing for a serological survey has to be based on a clear analysis of the objectives, that is whether the identification of animals exposed to the pathogen or of the animals with an active infection is desired.

Numerous studies have shown that different genospecies of the *B. burgdorferi* s.l. complex manifest various degrees of preference towards specific vertebrate hosts (Kurtenbach et al. 2002); the crucial factor that explains this selective transmission and preferential association between reservoirs and *B. burgdorferi* s.l. genospecies was identified as the host’s complement system. The level of complement activity in the serum is an important intrinsic factor in determining reservoir competence of different vertebrates for *B. burgdorferi* s.l. genospecies (Isogai et al., 1994; Kurtenbach et al., 1998; Kuo et al., 2000; Nelson et al., 2000; Lane et al., 2006). This does not mean that the mere identification of strains resistant to a species’ complement equals the confirmation of reservoir competence for that species, the only definitive confirmation of this status is represented by tick xenodiagnosis.
The study described in chapter II.III.II. aimed at evaluating borrelicidal effect of horse serum, based on the lytic action of the complement, as a first step in investigating the reservoir potential of this species, as well as underlining any differences in sensitivity between genospecies, strain type or passage number.

The utilized protocol was adapted after the one described by van Dam et al. (1997); 6 strains of *B. burgdorferi* s.l. were used, representing different genospecies, and passage numbers. Of these, 2 were reference strains (ATCC *B. burgdorferi* s.s. and ATCC *B. garinii*, LGC Standards, UK), while the others were wild strains, isolated from ticks in South Moravia, Czech Republic (*B. burgdorferi* s.s. CB53, *B. garinii* CB61, *B. afzelii* CB43/VIII și *B. afzelii* CB43/XI). The utilized sera originated from horses negative for IFA testing. Two controls were used: M1 was a mix of 50% culture with 50% heat inactivated sera, kept for 30 minutes at 56°C in order to abolish the effect of the complement, while M2 was represented by 100 µl of each culture, with no added serum. The number of motile spirochetes in each sample, including controls, was evaluated after 24h.

The changes in the numbers of motile bacteria during the 24h of observation is illustrated in figure 4. A strain was considered sensitive to the borrelicidal effect of horse serum if the number of spirochetes from the 50% serum sample was significantly lower compared to the number found in heat treated control and culture control (one-way ANOVA, post hoc Dunnett test, p < 0.05). These conditions were met by 3 strains, the other 3 (ATCC *B. burgdorferi* s.s., ATCC *B. garinii* and *B. afzelii* CB43/VIII) being considered resistant.

In conclusion, it can be established that the sensitivity of *B. burgdorferi* s.l. strains to complement mediated lysis of horse serum varies within one genospecies, with the reference strains proving to be more resistant in this study. A high number of passages can have an adverse effect on the resistance of *B. burgdorferi* s.l. strains to the complement mediated killing, a fact that warrants the use of low passage strains in complement sensitivity experiments.

Chapters II.IV.I. and II.IV.II. focused on the role of the wild boar (*Sus scrofa*) in sylvatic transmission cycles of Lyme borreliosis. Wild boar is a widespread game species in Europe, and besides wild ruminants, it represents a suitable host for ticks, especially adult stages. The studies were meant to evaluate the wild boar's role both as a reservoir and as a sentinel species.

The objective of chapter II.IV.I. was to assess the value of wild boar as a sentinel species, as appreciated by molecular detection of *B. burgdorferi* s.l. in organs harvested from hunted animals. The prevalence of *B. burgdorferi* s.l. according to county and sampling period was evaluated for the identification of spatio-temporal differences in spread. The results were correlated with those obtained from the National Center for Surveillance and Control of Communicable Diseases (CNSCBT) regarding the number of confirmed Lyme disease cases in humans for each county in 2011.
Figure 4. Dynamic evolution of the number of motile spirochetes in samples exposed to different serum concentrations, and in the controls. (a) *B. burgdorferi* s.s. CB53; (b) ATCC *B. burgdorferi* s.s.; (c) *B. garinii* CB61; (d) ATCC *B. garinii*; (e) *B. afzelii* CB43/XI; (f) *B. afzelii* CB43/VIII.

Between 2007 - 2008 and 2010 - 2012, 870 samples from 16 counties were analyzed using a classic PCR protocol. The samples consisted of spleen, liver, kidney, lung and lymph node fragments. The genetic material of *B. burgdorferi* s.l. was identified in 24 of these samples, the global prevalence of the pathogen in the studied wild boar populations from Transylvania being 2.76%. Detailed results are described in table 3.
Table 3. Distribution of wild boars infected with *B. burgdorferi* s.l. according to counties and sampling period

<table>
<thead>
<tr>
<th>County</th>
<th>2007-2008 Prevalence (n=435) n(%)</th>
<th>County</th>
<th>2010-2012 Prevalence (n=435) n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alba</td>
<td>8/29 (27.59)*</td>
<td>Alba</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>Arad</td>
<td>0/19 (0)</td>
<td>Arad</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td>Bihor</td>
<td>0/3 (0)</td>
<td>Bistrița Năsăud</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Brașov</td>
<td>0/10 (0)</td>
<td>Brașov</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>Cluj</td>
<td>2/27 (7.41)</td>
<td>Cluj</td>
<td>2/32 (6.25)</td>
</tr>
<tr>
<td>Covasna</td>
<td>0/62 (0)</td>
<td>Covasna</td>
<td>0/33 (0)</td>
</tr>
<tr>
<td>Hunedoara</td>
<td>0/33 (0)</td>
<td>Caraș Severin</td>
<td>0/23 (0)</td>
</tr>
<tr>
<td>Harghita</td>
<td>1/51 (1.96)</td>
<td>Harghita</td>
<td>0/27 (0)</td>
</tr>
<tr>
<td>Maramureș</td>
<td>0/11 (0)</td>
<td>Maramureș</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>Mureș</td>
<td>2/32 (6.25)</td>
<td>Mureș</td>
<td>0/34 (0)</td>
</tr>
<tr>
<td>Sibiu</td>
<td>7/101 (6.93)</td>
<td>Sibiu</td>
<td>0/72 (0)</td>
</tr>
<tr>
<td>Satu Mare</td>
<td>1/43 (2.33)</td>
<td>Satu Mare</td>
<td>1/32 (3.13)</td>
</tr>
<tr>
<td>Timișoara</td>
<td>0/6 (0)</td>
<td>Sâlaj</td>
<td>0/16 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Timișoara</td>
<td>0/35 (0)</td>
</tr>
</tbody>
</table>

Regarding the spatial distribution of the pathogen, the recorded prevalences were statistically equal in all counties, except for the samples obtained from Alba county in the 2007-2008 period. These samples showed an infection rate of 27.59%, significantly higher than the values recorded in other counties (p = 0.03). This difference did not persist in the 2010-2012 sampling period. Significant differences were also observed between prevalences recorded for the 2 sampling periods (p = 0.002). No correlation was identified between human Lyme disease cases and positive wild boar samples for the 2010-2012 sampling period ($r_s = -0.22, p = 0.4$), or both periods combined ($r_s = 0.22, p = 0.39$).

In conclusion, it was shown that *B. burgdorferi* s.l. can cause a systemic infection in wild boar, demonstrated by the possibility of molecular identification of genetic material belonging to the spirochete in the animal’s internal organs, but the prevalence does not reflect the potential risk for humans, translated in number of Lyme disease cases in a certain area, thus, the value of the wild boar as a sentinel species is low.

The reservoir potential of the wild boar has not been studied up to the present; it is not established if the role played by this animal in the sylvatic cycles is the one of an amplifying host or, on the contrary, of a zooprofilactic host. The experiment described in chapter II.IV.II. aimed to evaluate the complement-based borrelicidal activity exhibited by
wild boar serum, against different strains of \textit{B. burgdorferi} s.l., as a first step in confirming or ruling out the reservoir status of this species.

Three strains of \textit{B. burgdorferi} s.l. belonging to 2 genospecies were used, of these 2 were represented by reference strains (ATCC \textit{B. burgdorferi} s.s. and ATCC \textit{B. garinii}, LGC Standards, UK), while the third one was a wild strain (\textit{B. burgdorferi} s.s. CB53). Neither strain had a passage number greater than 10. Sera was obtained from 3 wild boars shot on Band hunting ground (Mureș county). The controls were the same ones described in chapter II.III.II.; the number of motile spirochetes was evaluated after 24 h (Fig. 5).

Wild boar sera was borrelicidal for all 3 tested strains, which suggests that the role of this species in sylvatic transmission cycles of Lyme borreliosis may be similar to that of the wild ruminants, but to confirm this hypothesis testing of a much larger number of strains, as well as tick xenodiagnostic studies are necessary.

\textbf{Figure 5.} Dynamic evolution of the number of mobile spirochetes in samples exposed to different wild boar serum concentrations, and in controls. (a) \textit{B. burgdorferi} s.s. CB53; (b) ATCC \textit{B. burgdorferi} s.s.; (c) ATCC \textit{B. garinii}
References


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Summary


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