Serological investigation of *Borrelia burgdorferi* sensu lato infection in dogs, in Southern Romania

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Abstract. Human and canine Lyme borreliosis (LB) are tick-borne diseases currently emerging in Europe. Continuous monitoring of exposure rates to the causative agent *Borrelia burgdorferi* sensu lato in dog populations allows an early recognition of the geographic expansion of endemic areas, where both humans and dogs are at risk. Little is known in Romania on LB infection rates and the potential risks for the animal and public health. Therefore, a serological screening was carried out to investigate the exposure to *B. burgdorferi* s.l. infection in dogs in Southern Romania. In order to accomplish this, 186 dogs originated from four counties (Ilfov, Calarasi, Teleorman, Valcea) in Southern Romania were included in the study. All the dogs were presented for the spaying procedure between March and May 2017, of which 23 were pure breed dogs and 163 mixed breed. The dogs were clinically healthy, aging from 8-months to 14-years, of different life-categories [stray dogs (n=93), owned dogs (n=69), and shelter dogs (n=24)], all with outdoor access. Serum samples were collected from all dogs and investigated in a two-step testing procedure. In the first step, all samples were analyzed for IgG antibodies using a commercial ELISA test; then, the positive and/or borderline samples were subjected for further confirmation to a Western Blot (WB) commercial test which is also able to discriminate between exposure and acute infection based on positive reaction to one and/or many specific antigens (VlsE, p100, p39, OspA, OspC, p21, p18). WB confirmation was established based on positive band to VlsE alone or two other bands. Overall, six of the investigated dogs (6/185; 3.2%; 95% CI: 1.2 - 6.9) were seropositive by ELISA-testing, including five positive and one borderline. Of these, three samples (3/6; 50.0%) were further confirmed as positive by WB: two (2/3; 66.7%) were classified as acute infections and one (1/3; 33.3%) as indication of *B. burgdorferi* s.l. contact. The remaining three samples were classified as borderline. Statistical analysis showed no positive correlation between gender, age, breed, and lifestyle. These findings document the exposure of dogs to *Borrelia burgdorferi* s.l. infection and highlight the potential risks for both animal and public health in Southern Romania.

Keywords: *Borrelia burgdorferi* sensu lato; Lyme borreliosis; Serological survey; Dogs; Southern Romania.

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Introduction

Lyme Borreliosis (LB) is a common occurring tick-borne disease in the northern hemisphere caused by Gram negative bacteria of the *Borrelia burgdorferi* sensu lato complex. Annually 300,000 human cases are estimated in the USA and around 85,000 cases in Europe (Pritt et al., 2016). The disease’s incidence is influenced by the abundance rate and geographical distribution of the tick vector. In Europe, the tick species *Ixodes ricinus* is involved in the transmission of *B. burgdorferi* s.l., thus its presence and the identification of the causative agent in this tick is considered a risk indicator for a given area (Guerra et al., 2001).

Dogs are often first to come in contact with ticks due to their inquisitive behavior and ability to harbor them in the fur. Given that, they are indicated as effective sentinels for LB (Irwin et al., 2017). Therefore, in Europe and the USA, numerous serological surveys have been conducted on dogs as an auxiliary of surveillance for human LB.

In Europe, among the 20 subspecies described (Clark et al., 2014), *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, *Borelia bavariensis* and *Borrelia spielmanni* are recognized as causative agents of LB in humans. Depending on the causative species and the clinical stage of the disease, a variety of clinical signs have been described in humans, including: *erythema migrans rash*, cranial nerve paralysis, carditis, monoarticular arthritis, and very rarely, encephalopathy or polyneuropathy (Wormser et al., 2016).

In dogs, *B. burgdorferi* s.l. infection usually results in low incidence of clinical disease, by comparison. Dogs with subclinical infections may be in a premunitive stage that could be protective, at least for the infecting strain (Littman et al., 2018). However, infections with multiple *Borrelia* species and/or infection with other pathogens may predispose dogs to clinical disease. In the latter case, syndromes characterized by polyarthritis and glomerulopathy have been associated with LB (Littman et al., 2006). Additionally, unspecific signs, such as fever, fatigue and limping have also been registered. Serological testing is the recommended method to document exposure to *B. burgdorferi* s.l. infection in dogs (Littman et al., 2018).

In Romania, due to rapid increase of the LB incidence in the last decades, particularly compared to the year 2009, the National Institute of Public Health has started in 2010 to report the epidemiological situation of human LB. Therefore, the number of confirmed LB cases between 2010 and 2015 varied from 265 to 698 cases/year (http://www.cnsctb.ro/index.php/analiza-date-supraveghere/boala-lyme-1).

Also, in Romania, *I. ricinus* is reported as the most common tick species; its distribution and the diversity of the harbored pathogens have recently been reported (Mihalca et al., 2012; Ioniță et al., 2013; 2016).

Continuous monitoring of exposure rates to the LB-causative agents *B. burgdorferi* s.l. in dog populations allows an early recognition of geographic expansion of endemic areas where both humans and dogs are at risk. Therefore, the aim of the present study was to investigate the exposure to *B. burgdorferi* s.l. infection among dogs, in Southern Romania.

Materials and methods

Animals and sampling

During spaying campaigns conducted in 2017, a total of 186 dogs, including stray (n=93), shelter (n=24), and owned dogs (n=69), were selected for a serological study to investigate their exposure to *B. burgdorferi* s.l. infection.

The dogs originated from four counties in Southern Romania: Ilfov (n=60), Calarasi (n=44), Teleorman (n=40) and Vâlcea (n=42). General information about the dogs, such as breed, gender, age, and tick infestation history has been recorded, as the owners provided (table 1).

All dogs were subjected to a clinical examination. During physical examination, all the found ticks were collected and preserved in 70% ethanol, for further species identification, using morphological keys (Estrada-Pena et al., 2004).
Dog blood samples were collected in clot activator tubes. Serum was extracted within hours after blood collection and kept frozen until processing for serological screening.

**Serological testing**

Serum samples were subjected to a two-step testing procedure (Robertson et al., 2000). In the first stage, serum samples were tested using an ELISA screening commercial test (Anti-Borrelia ELISA Dog - IgG kit; EUROMMUN AG, Germany). The test detects all relevant *Borrelia* species, using antigen extracts of *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. Determination of positive samples was based on the measurement of the IgG antibodies titer which had to be higher than 0.317 as recommended by the manufacturer.

For further confirmation, the sero-reactive samples from the first step were subjected to the second stage testing, using a Western Blot commercial kit (Anti-Borrelia EUROLINE Dog: EUROMMUN AG, Germany). This test allows to simultaneously detect seven different *Borrelia*-specific antibodies using highly specific recombinant antigens: VlsE-Bb (purified recombinant antigen from *Borrelia burgdorferi*; p100 (purified recombinant protein p100); p41 and p39 (purified recombinant p41-flagellin and p39-bmpA); p31 (purified recombinant OspA); p25 (OspC); p21; p18. Based on the type and number of positive antigen bands, the test also allows differentiation between vaccination and infection.

The testing procedure is briefly described here: test strips coated with purified antigens (immunoblot strips) were firstly incubated with the diluted serum samples; during the incubation, in positive samples, the specific IgG Ab bind to the corresponding antigenic site. Thereafter, a second incubation was carried out to detect the bound antibodies using an enzyme conjugate (enzyme-labeled anti-dog IgG) that catalyses a color reaction. After stopping the reaction, the incubated membrane strips were dried and photographed. EUROMMUN AG and EUROLLineScan software were used for automate evaluation and detailed documentation of results. The software identified the bands, measured their intensity and based on the signal intensity, automatically provided the final results for each sample, such as: no signal [0]: negative; very week or borderline band [(+)]: borderline; medium to strong signal [+]: positive. Thereafter, based on the type and number of antigen band reaction, the results were classified, according to the manufacturer's protocol, as follows: (i) VlsE positive: classified as positive and serological indication of infection; (ii) both VlsE and OspA negative but ≥2 other bands positive: classified as positive and serological indication of contact; (iii) VlsE negative but OspA plus ≥1 band positive: classified as positive and indication of contact and/or immunization; (iv) only OspA positive: classified as positive and; immunization; (v) 1 band positive (except OspA and VlsE): classified as borderline; (vi) ≥2 bands borderline: classified as borderline; (vii) no bands or week bands: classified as negative.

**Statistical analysis**

Quantitative Parasitology 3.0 software was used to analyze the correlations between different variables (Rozsa et al., 2000).

**Results**

Between March and May 2017, altogether 186 dogs originating from four counties in Southern Romania were randomly selected for a serological screening to investigate the exposure to *B. burgdorferi* sl. infection. All dogs had access to urban green areas in their originating region and no one has had a history of travelling outside of Romania, based on data provided by the owners.

Dogs included in the present study consisted of 24 males and 162 females, 23 were pure-bred (six different breeds: Shepherd raven, Pekingese, Westie, Cocker, Bichon and German Shepherd) and 163 were mixed-bred. The dogs were aged between 8 months and 14 years [average 2.7 years; standard deviation (SD) 2.4]). All dogs were declared clinically healthy during the clinical examination.
A number of 30 ticks were collected from a total of nine dogs during the clinical evaluation. The tick specimens belonged to three tick species: *I. ricinus* (n=1; one female), *Dermacentor marginatus* (n=9; 7 females and 2 males) and *Rhipicephalus sanguineus* (n=20; 12 females, 8 males).

Subsequent to the serological testing, six of the investigated dogs (3.2%; 95% CI: 1.2 - 6.9) were seropositive by ELISA-testing of which five showed a clear positive response and one was classified as borderline (table 1).

**Table 1.** Serological prevalence of *Borrelia burgdorferi* sensu lato infection, by using a two-step testing procedure (ELISA and Western Blot), among dogs in Southern Romania

<table>
<thead>
<tr>
<th>Number of <em>Borrelia burgdorferi</em> s.l. seroreactive dogs (%)</th>
<th>ELISA test</th>
<th>Western Blot test*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Positive</td>
</tr>
<tr>
<td>Total</td>
<td>186</td>
<td>6 (3.2)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>162</td>
<td>6 (3.7)</td>
</tr>
<tr>
<td>male</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 1 year</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>1 - 8 years</td>
<td>153</td>
<td>6 (3.9)</td>
</tr>
<tr>
<td>&gt;8 years</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Breed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mixed</td>
<td>163</td>
<td>6 (3.7)</td>
</tr>
<tr>
<td>pure breed</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Tick infestation***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>177</td>
<td>6 (3.4)</td>
</tr>
<tr>
<td>Life Style</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stray</td>
<td>93</td>
<td>3 (3.2)</td>
</tr>
<tr>
<td>Shelter</td>
<td>24</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>Owned</td>
<td>69</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Area (County)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ilfov</td>
<td>60</td>
<td>2 (3.3)</td>
</tr>
<tr>
<td>Teleorman</td>
<td>40</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>Călărași</td>
<td>44</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td>Vâlcea</td>
<td>42</td>
<td>1 (2.4)</td>
</tr>
</tbody>
</table>

* the positive and/or borderline ELISA samples were subjected to further Western-Blot confirmation

**the prevalence value (%) is reported to the total number of animals for each category

*** at the moment of sampling.
Of the six ELISA positive samples that were further subjected to the second step: WB testing, three (1.6%; 95% CI: 0.3–4.7) were confirmed as *B. burgdorferi* s.l. positive. The other three (Id 3, 5, 6) were WB classified as borderline; these have also reacted to important recombinant proteins in the WB test such as: OspC, p41, p21, p100 and VlsE. However, the intensity of the reaction was considered insufficient for a positive result. The sample classified as borderline after the ELISA step, was also identified as borderline after the WB step (Id 6) (figure 1).

The three confirmed *B. burgdorferi* s.l. infected dogs (Id 1, 2, and 4) (figure 1) originated from three different counties: Ilfov, Teleorman, and Vâlcea. Of these, two dogs (66.7%) were classified with acute infections, and one (33.3%) with indication to *B. burgdorferi* s.l. contact. All positive dogs were females of 2, 2.5, and 3 years-old, respectively (table 1). The statistical analysis showed no positive correlation between gender, age, breed, or lifestyle of the dogs.

**Figure 1.** Western Blot evaluation and interpretation of *Borrelia burgdorferi* s.l. infection in dogs, based on the positive reaction to one and/or many specific antigens (each strip shows antigens and their arrangement: VlsE, p100, p39, OspA, OspC, p21, p18, Control)

**Discussion**

A two–step testing procedure by means of ELISA and WB assays was used in this study to investigate the exposure to *B. burgdorferi* s.l. infection of dogs. Both the WB and the ELISA test are specifically designed to detect *B. burgdorferi* s.l. infection acquired in Europe, knowing the considerable differences due to the causative genospecies.
The exposure to *B. burgdorferi* s.l infection of dogs from Southern Romania was documented by evaluating the IgG titer and antibodies types in a two-step procedure. The presence of robust positive bands for the VlsE-Bb recombinant protein demonstrates natural infection of the positive dogs (Baum et al., 2014). A strong reactivity was also registered to OspC (which is commonly used as late stage infection marker) and to p41. The absence of reactivity to OspA (which is an indicator of immunization) confirms that vaccination against LB is not yet practiced in Romania, given the autochthonous origin of dogs.

The presence of ticks on dogs was not correlated with the sero-reactive *B. burgdorferi* s.l. dogs. This was expected, knowing that a period of weeks is needed for occurrence of circulating IgG antibodies, in the case of bacteria transmission (DeBiasi, 2014).

The final overall prevalence of confirmed *B. burgdorferi* s.l. infections in the tested dogs in this study was 1.6% (3/186). Similar rates of *B. burgdorferi* s.l. infection in dogs have been reported in other European countries, such as: Germany (1.9–10.3%), Portugal (8.5%), Slovakia (2.8%), and Bulgaria (2.4%) (Krupka et al., 2007; Čabanová et al., 2015; Pantchev et al., 2015; Alho et al., 2016). The highest infection rate in Europe was reported for pet and hunting dogs in the Netherlands, varying between 17.0% and 18.0% (Goossens et al., 2001) and eastern Poland, of 11.0% (Dziegiel et al., 2015).

In Romania, *B. burgdorferi* s.l. infection in dogs has been reported with different prevalence rates varying from 0.56% (using SNAP 4DX test) to 6.52% (by ELISA and IFA tests) (Kiss et al., 2011; Anghel et al., 2016). Additionally, *B. burgdorferi* s.l. infection was also reported in horses with a prevalence of 11.92% (Kiss et al., 2011).

When using dogs as sentinels for *B. burgdorferi* s.l. infection in endemic areas, it is expected to have higher infection prevalence than in human populations. Seroprevalence values higher than 5% for canine populations are considered a sensitive marker of human risk whereas a seroprevalence lower than 1% is considered an indicator of a rather low risk of infection (Mead, 2011).

In human populations, in Romania, the mean incidence of reported LB is rather low compared to the European values (Rizzoli et al., 2011). A study implying 2,500 healthy blood donors and forestry workers from different areas of Romania reported an overall seroprevalence of 4.3% in healthy blood donors and 9.3% in forestry workers (Hristea et al., 2001).

Variable positive rates to *B. burgdorferi* s.l. infection, in both human and dog populations are not a surprise since tick populations and tick bites in Romania have been reported more often, lately. Recently, several studies reported the prevalence of *B. burgdorferi* s.l. in ticks originating in different areas of Romania, ranging from 3.8%, 18.0% to 25.0% (Coipan and Vladimirescu, 2011; Kalmar et al. 2013; Răileanu et al. 2017), depending on the geographical area. This data sustains the existence of localized foci of *B. burgdorferi* s.l. in Romania that include small regions and specific microclimates, data previously confirmed in ecological studies (Ioniță, 2003; Ioniță et al., 2010; Andersson et al., 2018), highlighting the need for a proper surveillance of the LB dynamics in both human and animal populations (Mitrea, 2002).

**Conclusion**

This study provides new evidence of exposure to *B. burgdorferi* s.l. in dog populations from Southern Romania. Therefore, further monitoring studies of infection dynamic for early recognition of its spreading in both dog and human populations are planned together with informative campaigns regarding the importance of preventative measures concerning tick bites and the associated risks for *B. burgdorferi* s.l infection.
References


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