Antibodies to *Borrelia turicatae* in experimentally-infected dogs cross-react with *B. burgdorferi* serologic assays.

Jenna R. Gettings*, Job E. Lopez*, Aparna Krishnavahjala, Brittany A. Armstrong, Alec T. Thompson, and Michael J. Yabsley

*Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA, USA. (JRG - jenna.gettings@uga.edu, ATT – alec.thompson@uga.edu, MJY – myabsley@uga.edu)

*Department of Pediatrics, National School of Tropical Medicine, Baylor College of Medicine, Houston, TX, USA (JEL - job.lopez@bcm.edu, AK – Aparna.Krishnavahjala@bcm.edu)

*Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, USA (BAA – Brittany.Armstrong@bcm.edu)

*Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA, USA.

Running Title: *Borrelia turicatae* antibody cross-reactivity

#Address correspondence to Jenna R. Gettings, jenna.gettings@uga.edu
Tick-borne relapsing fever (TBRF) is caused by several *Borrelia* spp (including *Borrelia turicatae*), which are primarily transmitted by *Ornithodoros* ticks. Relapsing fever group species are found worldwide except for Antarctica. Approximately 500 human cases were reported between 1990-2011 in the United States (likely an underestimate), while cases in domestic and wild dogs were reported from Florida, Texas, and Washington. TBRF spirochetes are related to *Borrelia burgdorferi*, the agent of Lyme borreliosis. Dogs are routinely screened for *B. burgdorferi*, but it is unknown if infection with TBRF agents produce antibodies cross-reactive with *B. burgdorferi* assays. These data are critical for accurate surveillance of TBRF and Lyme borreliosis in dogs. In this study, *B. burgdorferi*-negative dogs were inoculated with *B. turicatae* and seroconversion was confirmed by the rBipA Western blot. Seropositive samples were tested with commercial and veterinary diagnostic laboratory *B. burgdorferi*-based tests. *Borrelia turicatae*-seroreactive samples cross-reacted with a whole cell IFA and two multi-antigen tests; but not with single antigen tests using C6. Cross-reactivity with TBRF can confound epidemiology and surveillance efforts, and confuse recommendations made by veterinarians for prevention and control. These findings demonstrate the need to critically evaluate results of *B. burgdorferi* diagnostic tests in the context of the assay type, the animal’s geographical location, and history of travel as well as highlighting the need for commercially-available specific diagnostic tests for TBRF spirochetes.

**Keywords:** *Borrelia burgdorferi, Borrelia turicatae, Lyme borreliosis, Tick-Borne Relapsing Fever*
INTRODUCTION

Phylogenetically, the *Borrelia* genus can be separated into two major clades, the Lyme borreliosis group, which includes the causative agents of Lyme borreliosis, and the relapsing fever group (1). In North America, tick-borne relapsing fever (TBRF) is caused by *B. miyamotoi*, *B. hermsii*, *B. parkeri*, and *B. turicatae* (2). TBRF spirochetes of medical importance in the United States were thought to be transmitted by only argasid ticks (soft ticks) with *Ornithodoros hermsii*, *O. parkeri*, and *O. turicata* transmitting *B. hermsii*, *B. parkeri*, and *B. turicatae*, respectively (3). However, recently, some hard tick-transmitted relapsing fever spirochete species have been discovered (e.g. *B. miyamotoi* is transmitted by *Ixodes* spp. and *B. lonestari* is transmitted by *Amblyomma americanum*) (4,5).

Despite the divergence between the Lyme borreliosis and relapsing fever groups, case reports of TBRF in humans and dogs have suggested that antibodies against the relapsing fever spirochetes cross-react with *B. burgdorferi* sensu stricto (henceforth *B. burgdorferi*) (1,6–9). Unfortunately, the available histories from these case reports cannot completely rule-out exposure to *I. scapularis* and thus cannot rule out exposure to *B. burgdorferi*. This is especially true for human cases of *B. miyamotoi*, a relapsing fever spirochete transmitted by *I. scapularis* (10). Similarly, exposure to vectors of *B. burgdorferi* cannot be ruled out in the current case reports of TBRF in dogs. As a result, any cross-reactivity reported in these cases may be a result of prior exposure to *B. burgdorferi*, and not true cross-reactions with antibodies against relapsing fever spirochetes.
Borrelia serological testing in dogs is commonly used to determine exposure to \textit{B. burgdorferi} and to diagnose Lyme borreliosis when used in conjunction with other tests, including the detection of proteinuria and quantitative tests for antibodies against the C6 peptide (11).

However, TBRF spirochetes can be transmitted by the same vector of \textit{B. burgdorferi}, as in the case of \textit{B. miyamotoi} in the Northeast (10), and the distribution of \textit{Ornithodoros} spp. vectors of relapsing fever spirochetes can overlap with \textit{I. pacificus} (2,12). Thus, cross-reactivity or co-infections may occur. Epidemiological studies on the prevalence of \textit{B. burgdorferi} exposure will be confounded and measures of disease frequency will be difficult to interpret. The objective of this study was to evaluate serological responses from dogs infected by needle inoculation with \textit{B. turicatae}. These animals were confirmed to be seronegative for \textit{B. burgdorferi} and after they seroconverted to \textit{B. turicatae}, we assayed samples with commercial or reference laboratory \textit{B. burgdorferi} diagnostic tests to determine serological cross-reactivity to \textit{B. burgdorferi} diagnostic antigens. These findings demonstrate the need to critically evaluate the results of \textit{B. burgdorferi} diagnostic tests in the context of the assay type, the animal’s geographical location, and travel history, and highlights the need for commercially-available diagnostic tests specific for TBRF spirochetes.

**MATERIALS AND METHODS**

**Experimental Animals**

Seven 8-month old laboratory raised beagles (3 female and 4 male) (Ridglan Farms, Inc.) were individually housed in a climate-controlled and tick-free animal facility at the University of
Georgia (UGA) (Athens, GA). A commercial dry dog food diet was provided daily and water available ad libitum. Dogs were vaccinated using standard core canine vaccines. All methods were reviewed and approved by UGA’s Institutional Animal Care and Use Committee (A2017 04-005).

All pre-inoculation serum samples were tested for antibodies to *B. burgdorferi* on SNAP® 4Dx® Plus (IDEXX Laboratories, Westbrook, ME) and VetScan® Canine Lyme Rapid Test (Abaxis, Union City, CA). Both commercial tests were performed according to manufacturer’s instructions. Pre-inoculation samples were also tested with the recombinant *Borrelia* immunogenic protein A (rBipA) Western blot (WB) (see below).

**Inoculation**

Dogs were inoculated with a low passage (p9) *B. turicatae* strain 91E135 that was originally obtained from *O. turicata* in Texas (13). Dogs received one of three doses: 100 spirochetes (1 dog), 250 spirochetes (3 dogs), and 500 spirochetes (3 dogs). The culture was diluted with BSK H media with 6% rabbit serum (Sigma-Aldrich, St. Louis, MO) to the appropriate dose in 0.5 mL aliquots using a haemocytometer under light microscopy. During the counting and dilution process, spirochete viability was confirmed by examination of spirochete motility. One half (0.25 mL) was inoculated subcutaneously between the shoulder blades where a 2 inch by 2 inch area had been shaved to allow visualization of potential skin reactions, and the remaining half was inoculated intradermally to mimic a tick bite. Two dose routes were used to maximize chances of infection since experimental exposure using cultured spirochetes route has not been investigated.
with *B. turicatae*, and route of exposure has been shown to be an important factor for some vector-borne pathogens (14).

**Observation and Sample Collection**

Dogs were observed daily to assess their attitude, appetite, and activity level. Lymph nodes were palpated, and rectal temperature was also taken daily. Blood was collected prior to inoculation on -2 days post-inoculation (DPI), 29 DPI to assess seroconversion, and 43 DPI to compare the serology tests. Serum was obtained by collecting blood into serum collection vacutainers and spinning prior to freezing. Serum samples were stored at -20°C until use.

**Confirmation of Seroconversion to *B. turicatae***

Seroconversion to *B. turicatae* was evaluated by immunoblotting using *B. turicatae* rBipA, as previously described (15). Serum samples collected at -2 and 29 DPI were used at a dilution of 1:200. The secondary molecule was Rec-protein G-HRP (ThermoFisher Scientific, Waltham, MA, USA) diluted 1:4,000. Serological reactivity was determined by chemiluminescence using the Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

**B. burgdorferi Serologic Assays Evaluated and Criteria for Test Interpretation**
Cross-reactivity of anti-*B. turicatae* antibodies with *B. burgdorferi* antigens and assays was assessed using commercially available patient-side tests and by submitting samples to reference diagnostic labs. Two patient-side rapid tests were assessed: SNAP® 4Dx® Plus (IDEXX Laboratories, Inc Westbrook, ME USA) and VetScan® Canine Rapid Lyme Test (Abaxis, Union City, CA USA). Three reference laboratory tests were assessed: Lyme Quant C6® (IDEXX Laboratories, Inc Westbrook, ME USA), *Borrelia burgdorferi* Titer (Lyme Disease) indirect fluorescent antibody (IFA) test (Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX USA), and Accuplex®4 (Antech Diagnostics, Fountain Valley, CA USA).

Aliquots of sera from 43 DPI were used for all the listed tests. VetScan® was performed following the manufacturer's instructions with newly opened kits prior to their expiration dates (16). For the remaining tests, samples were submitted to the respective laboratories for analysis.

SNAP® 4Dx® Plus and Lyme Quant C6® both detect antibodies reactive to C6, a synthetic peptide based on the invariable region 6 of the surface membrane protein VlsE (17). The former is a qualitative test that is interpreted as positive when both color is visible in the sample spot and there is color development in the positive control spot (18). The latter is a quantitative C6 ELISA. Antibody levels > 30 U/mL are reported as a high C6 antibody level; values less than 30 U/mL are interpreted as low C6 antibody levels (19). It is useful to note that diagnostically, Lyme Quant C6® is not recommended unless a sample is positive on the SNAP® test.
VetScan® Canine Rapid Lyme Test detects antibodies reactive to VlsE, OspC, and p41 (flagellin) (20). Samples are reported as positive when there is any color development of the test line and color development of the control line.

Accuplex®4 detects antibodies against *B. burgdorferi*, *Ehrlichia canis*, *Anaplasma phagocytophilum*, and an antigen from *Dirofilaria immitis*. The *B. burgdorferi* antigens included in the test are OspA, OspC, OspF, P39, and small Lyme peptide (SLP), a proprietary synthetic peptide (21). Antibody levels are measured quantitatively but are not reported. A proprietary algorithm is used to assign results based on the values (22). The laboratory reports samples as either negative or positive for vaccination and then either negative to exposure/infection or positive for early or late exposure/infection antibodies to *B. burgdorferi*.

The whole cell IFA tests serum using two-fold dilutions of 1:64 to 1:2048 and does not differentiate between vaccination and natural infection (23). Laboratory reports the lowest positive dilution. Titers < 64 are interpreted as negative for antibodies against *B. burgdorferi*.

**RESULTS**

Prior to inoculation, dogs were confirmed to be seronegative to both *B. burgdorferi* and *B. turicatae* using the SNAP® 4Dx® and VetScan® assays for *B. burgdorferi* and the rBipA WB for *B. turicatae*. Infection was also confirmed by PCR and blood culture (data not shown).
No dog developed clinical signs (lethargy, inappetence, fever, or lameness) of relapsing fever during their daily checks. Based on rBipA WB testing, six of the seven dogs developed antibodies to *B. turicatae* by 29 DPI.

**Serologic testing using *B. burgdorferi* assays**

Of the six dogs that sero-converted to *B. turicatae*, five tested positive on at least one of the tests (Table 1). Of the five tests evaluated, three were found to react to anti-*B. turicatae* antibodies. The highest magnitude of cross-reactivity was noted for the whole cell IFA. Five of the six seroconverted dogs tested positive with a titer range of 256 to ≥ 2,048. The dogs with the highest titers (VVZ, QSY, and SYZ) also cross-reacted with other tests. Only one dog (SYZ) cross-reacted on more than two tests. The three most reactive dogs in the study (VVZ, QSY, and SYZ) had measurable antibody levels above 10 U/ml with the quantitative C6 ELISA. However, these results are below the positive threshold for this test (30 U/ml) so would have been reported as negative. Those three dogs also had color development on the test line of the VetScan® test. Any color development is considered positive per the product instructions. The test line for these three samples were fainter than the control line, with two that were readily visible, and the third very faint. The faint sample was deemed equivocal based on the assumption that the test would likely have been repeated in a clinical setting.

**DISCUSSION**
We report that antibodies produced in response to infection with *B. turicatae* 91E135 can cross-react with tests designed to detect *B. burgdorferi*, the causative agent of Lyme borreliosis. We chose the 91E135 strain of *B. turicatae* given high degree of genetic relatedness compared to other available *B. turicatae* isolates (13). Interestingly, the infectious doses used in the study were not associated with the reactivity of antibodies, as the dog that received the lowest dose exhibited the greatest cross-reactivity among the assays tested (3 of 5), though this sample is too small to measure this statistically.

The dogs in this study did not develop any physical examination signs of infection, raising questions about the possibility of subclinical infections with relapsing fever spirochetes in canines. Surveys of healthy dogs in known endemic ranges would help identify the presence of subclinical infections within the United States. There are estimates of the prevalence of exposure among dogs with suspected tick-borne illness. A recent serosurvey in Texas found 2% of samples submitted for tick-borne pathogen testing had antibodies to *B. turicatae* GlpQ as determined by WB (24). The prevalence of infection among a more generalized population found 0.68% of samples tested positive by qPCR from those submitted for any reason to a veterinary diagnostic laboratory (25). The history and clinical signs were unknown for those samples. Although neither study allows us to estimate the prevalence of subclinical infections, they do identify a larger number of exposed dogs than would be expected given the small handful of cases reported in the literature.

It is important to note that the ecology of *B. turicatae* and *B. burgdorferi* and their vectors vary. There is only limited overlap in the currently known geographic distribution of endemic canine
Lyme borreliosis (26) and the known presence of *Ornithodoros* spp. ticks in the United States, with the greatest areas of possible overlap occurring in the western United States between *Ixodes pacificus* (27) and *Ornithodoros* spp. (2,28). Despite this, many dogs test positive for *B. burgdorferi* exposure outside of the endemic region (29). Although these cases may result from travel-related exposure or sporadic translocation of *B. burgdorferi* vectors, cross-reactivity with relapsing fever spirochetes cannot be ruled out. In the western region, there are several relapsing fever spirochete species with a canine case of *B. hermsii* recently reported in Washington (30).

Most cases of *B. turicatae* have been reported from Texas and Florida (6–8). Although we did not test cross-reactivity with *B. miyamotoi*, this species may represent the greatest challenge diagnostically because it has the same vectors as *B. burgdorferi*, so co-exposure could be common (10). In regions where both relapsing fever and Lyme borreliosis may occur, veterinarians should be aware that cross-reactivity could occur and should perform appropriate tests for an accurate diagnosis. Veterinarians in the southern and western states that observe positive tests in dogs with no travel history to areas where Lyme borreliosis is considered endemic should strongly consider exposure to relapsing fever spirochetes.

There are some limitations to this study. First, we do not know the minimum infectious dose of *B. turicatae* to canines via needle inoculation, and so the doses used in this study may not have been large enough to induce physical signs of infection. Similarly, the effects of tick saliva in the establishment of infection and disease progression remains unknown and future studies should focus efforts on natural transmission. Moreover, our results are limited by the use of just one species of relapsing fever spirochetes spp., and commercial assays with multiple antigens, some of which are proprietary. This prevents us from identifying specific antigens responsible for
cross-reactivity. The lack of positive reactions on the C6 tests support the high specificity
reported (31). However, some of the dogs did have measurable C6 antibody levels with the
quantitative assay, albeit below the threshold for being considered positive. Levels may have
increased later in infection or without treatment. We were unable to determine this as testing was
performed at just one time point (43 DPI); additionally, testing was not performed until after the
termination of the study, a limitation that prevented us from testing later time points. Regardless,
this limited cross-reactivity coupled with studies reporting cross-reactivity with *B. miyamotoi*
and *B. hermsii* (32,33) raises the question as to whether a proportion of C6-seropositive animals
are exposed to relapsing fever spirochetes and not *B. burgdorferi*. Further work is required to
determine the seroprevalence of TBRF in healthy dogs throughout the US and the impact of
cross-reactivity on diagnosis of canine Lyme borreliosis. Of particular importance is potential
cross-reactivity with *B. miyamotoi*, which might affect surveillance of canine *B. burgdorferi*
seroprevalence within the range of *I. scapularis*.

Diagnosis of Lyme borreliosis in dogs is complicated by the low proportion of animals that
develop clinical disease after infection. Careful consideration of an individual dog’s history,
location with respect to Lyme borreliosis endemic regions, and appropriate testing will aid these
diagnoses, which may involve additional testing after a positive screening test (11). From the
results of this study, we find that whole cell IFAs are only useful for identifying exposure to
*Borrelia* spp. and should not be used to definitively diagnose Lyme borreliosis alone. For the
purposes of large-scale serosurveillance of *B. burgdorferi* in dogs in the United States, the C6
assay appears to be the best commercially-available assay of the ones tested, having the highest
specificity in this study. Definitive diagnosis of TBRF in dogs would be improved by the
availability of a serologic test (e.g. Western blot) using antigens specific to relapsing fever spirochetes; for example, glycerophosphodiester phosphodiesterase (GlpQ) and BipA in veterinary diagnostic laboratories (15,34).

Similar concerns of cross-reactivity have been reported for human infections of relapsing fever spirochetes and *B. burgdorferi* (10,12). Sera from patients with confirmed infections of *B. miyamotoi* have tested positive for antibodies against C6 and antibodies to antigens from both *B. miyamotoi* and *B. hermsii* have been shown to cross-react on *B. burgdorferi* ELISAs. However, naturally-acquired cases usually cannot rule out previous exposure to *B. burgdorferi*.

The results of this study highlight concerns about the use of certain antigens for Lyme borreliosis serologic tests and will help guide future research on cross-reactivity to specific antigens.

**Conclusion**

Dogs are frequently tested for exposure to *B. burgdorferi* either as a screening test during wellness examinations, or when tick-borne disease is suspected. We observed cross-reactivity between sera from *B. turicatae*-infected dogs and several commercially-available or reference laboratory *B. burgdorferi* serologic tests which may confound diagnosis and surveillance. Veterinarians practicing in areas where dogs may be exposed to relapsing fever spirochetes should be mindful of the possibility of cross-reactivity so that the appropriate recommendations are made for prevention and control of exposure. Epidemiologic studies should consider *Borrelia*
cross-reactions when measuring associations for risk factors and making recommendations on
tick prevention.

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Table 1. Compilation of all serological tests performed on samples collected 43 days post-inoculation for each *B. turicatae*-seropositive dog. (-) indicate a negative result, (+) a positive. Other information in the cell gives additional details about the results such as titers or the visual quality of the result for qualitative tests. Abbreviations: rBipA (recombinant *Borrelia* immunogenic protein A), WB (Western blot), IFA (indirect fluorescent antibody).

<table>
<thead>
<tr>
<th>Dog</th>
<th>Dose</th>
<th>rBipA</th>
<th>Whole cell IFA*</th>
<th>VlsE, OspC, p41b</th>
<th>OspA, OspC, P39, SLPc</th>
<th>C6d (qualitative)</th>
<th>C6e (quantitative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VVZ</td>
<td>High</td>
<td>+</td>
<td>+ (≥ 2,048)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>QSY</td>
<td>Medium</td>
<td>+</td>
<td>+ (≥ 2,048)</td>
<td>Equivocal</td>
<td>-</td>
<td>-</td>
<td>(13)</td>
</tr>
<tr>
<td>SYZ</td>
<td>Low</td>
<td>+</td>
<td>+ (1,024)</td>
<td>+ (faint)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SWZ</td>
<td>High</td>
<td>+</td>
<td>+ (256)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(13)</td>
</tr>
<tr>
<td>VQZ</td>
<td>Medium</td>
<td>+</td>
<td>+ (512)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(&lt; 10)</td>
</tr>
<tr>
<td>VAY</td>
<td>Medium</td>
<td>+</td>
<td>- (≤ 64)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(&lt; 10)</td>
</tr>
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</table>

*a* *Borrelia burgdorferi* Titer (Lyme Disease) indirect fluorescent antibody (IFA)  
b VetScan®  
c Accuplex®4  
d SNAP® 4Dx® Plus  
e Lyme Quant C6®
Erratum for Gettings et al., “Antibodies to *Borrelia turicatae* in Experimentally Infected Dogs Cross-React with *Borrelia burgdorferi* Serologic Assays”

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aSoutheastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA
bDepartment of Pediatrics, National School of Tropical Medicine, Baylor College of Medicine, Houston, Texas, USA
cDepartment of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, USA
dWarnell School of Forestry and Natural Resources, University of Georgia, Athens, Georgia, USA

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