Modified Two-Tiered Testing Enzyme Immunoassay Algorithm for Serologic Diagnosis of Lyme Disease

Khan, Farhan1,2; Allehebi, Ziyad1,2; Shabi, Yahya1,2; Davis, Ian1,2; LeBlanc, Jason1,2; Lindsay, Robbin3; Hatchette, Todd1,2

1Department of Pathology and Laboratory Medicine, Queen Elizabeth II Health Science Centre, Halifax, NS, Canada; 2Dalhousie University, Halifax, NS, Canada; 3National Microbiology Laboratory, Winnipeg, Manitoba, Canada.

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Contact information for the corresponding author:
Todd Hatchette
Division of Microbiology
Department of Pathology and Laboratory Medicine
Rm 315 MacKenzie Building
5788 University Avenue
Halifax, Nova Scotia B3H 1V8
Canada
Email: todd.hatchette@nshealth.ca

Contact information for the alternate corresponding author:
Farhan Khan
Division of Microbiology
Department of Pathology and Laboratory Medicine
Rm 326C MacKenzie Building
5788 University Avenue
Halifax, Nova Scotia B3H 1V8
Canada
Email: farhan.khan@nshealth.ca
Abstract:

The modified two-tier testing algorithm (MTTT) for Lyme disease (LD) has been approved by the FDA. Here we show that the MTTT detected 28% more cases of early infection compared to the standard two-tier algorithm while retaining high specificity in a region with a high incidence of Lyme disease.
Introduction:

Lyme disease (LD), or Lyme borreliosis, is the most frequently reported vector–borne disease in Canada [1] caused by the spirochete *Borrelia burgdorferi sensu lato* species complex that is transmitted to humans by infected blacklegged ticks. An increase in the number of locally acquired Lyme disease cases over the last decade has coincided with the northward geographic expansion of the range of blacklegged tick populations in southeastern and south-central regions of Canada. In 2018, 1,487 human cases of Lyme disease were reported in Canada [1]. The province of Nova Scotia is a risk area for Lyme disease with some regions having the highest rates of infection in Canada [1].

The clinical presentation of LD exists on a continuum divided into clinical stages. Early localized infection presents in about 80% of patients with erythema migrans (EM) [2]. However, if the rash is absent or left untreated *Borrelia burgdorferi* can disseminate throughout the body and appear as early disseminated infection, manifesting as multifocal EM rash, nonspecific influenza-like illness, arthralgia, meningitis, neuropathy, or carditis. Ultimately, late disseminated disease occurs if left untreated resulting in oligoarthritis of large joints and rarely neurologic disease.

In Canada, the current method for laboratory diagnosis of LD is detection of antibodies against *B. burgdorferi* using the standard two-tiered testing algorithm (STTT), which consists of an enzyme immunoassay (EIA) followed by IgM and/or IgG immunoblots (IB). The STTT is known to have poor sensitivity to detect early localized infection (<50%), but >99% sensitivity for detecting late infection [3]. Due to the high proportion of false negative results, early localized LD is usually diagnosed clinically. However, clinical diagnosis of early LD can be challenging, since some patients may not present with an EM rash or may have symptoms confused with other diseases [2]. Therefore, improving test sensitivity for early infection is crucial to permit definitive diagnosis of early LD and prevent consequences of untreated LD.

The first step in the STTT can include EIAs using *B. burgdorferi* whole-cell sonicate (WCS) or conserved synthetic peptides, such as the surface lipoprotein VlsE (variable major protein-like sequence, expressed) or C6 (invariable region 6 of VlsE) or C10 (conserved amino-terminal portion of outer surface protein C)
peptide [4]. Although, the specificity improves with conserved synthetic peptides EIAs compared to WCS, IBs are still required for optimal specificity. IBs are hampered by being time-consuming, having subjective scoring [4] and having limited sensitivity for early localized infection [3]. As well, the availability of IBs are geographically restricted in Canada to provincial laboratories in British Columbia and Ontario or the National Microbiology Laboratory (NML) in Manitoba.

Recently, the U.S. Food and Drug Administration (FDA) has approved a MTTT using two EIAs, which has been endorsed by the Centers for Disease Control and Prevention (CDC) and Infectious Diseases Society of America (IDSA) [5]. We recently showed a MTTT using a WCS EIA followed by C6 peptide EIA identified 25% more early LD cases than STTT, with a specificity of 99.56% [6]. The C6 EIA has not been approved for use in the MTTT by the FDA and Immunetics ended its production requiring us to validate two new EIAs for the MTTT. The FDA has approved an MTTT using the Zeus C10/VlsE followed by either the Zeus WCS total IgM/IgG or individual IgM and IgG EIAs. The aim of this study is to evaluate whether the new MTTT consisting of the Zeus C10/VlsE EIA followed by the Zeus WCS total IgM/IgG EIA would improve sensitivity to detect early LD without compromising specificity in Nova Scotia, Canada.

Methods:

From March to July 2020, all patient specimens submitted for LD serology that were positive or indeterminate on the Zeus C10/VlsE EIA total antibody (ZEUS ELISA Borrelia VlsE1/pepC10 IgG/IgM) were tested with the Zeus WCS EIA total antibody (ZEUS ELISA Borrelia burgdorferi IgG/IgM) (MTTT) in addition to IB testing at the NML in Winnipeg, Manitoba using the EUROIMMUN - Anti-Borrelia burgdorferi US EUROLINE-WB (IgG) and EUROMMUN - Anti-Borrelia EUROLINE-RN-AT-adv (IgM) ) kits (STTT). The Zeus WCS total IgM/IgG as the second-tier EIA was chosen over separated IgM/ and IgG EIAs for the improved laboratory efficiency of using only a 2-step process verses a 3-step process. All samples were submitted by clinicians as part of clinical management and testing was done prospectively.
A retrospective chart review using a standardized data collection tool was used to classify patients as “true LD” and identify the stage of infection. The data obtained included demographics, clinical findings that prompted the serologic test, consultations, and treatment. All data were deidentified, and patients were classified as having “true LD” if they had: 1) a positive IgG IB, 2) a negative IgG and positive or negative IgM IB, but with signs or symptoms consistent with early LD (e.g., EM rash or influenza-like illness (ILI) defined as fevers, with myalgias, fatigue or arthralgias), or 3) evidence of seroconversion between consecutive specimens. All patients with positive IgM and negative IgG IBs required compatible clinical syndromes to be categorized as true LD cases since IgM IB can be falsely positive. Patients who did not meet these criteria were considered false positive cases.

As an additional assessment of specificity, a second set of sera including 60 healthy individuals collected as part of a previous serosurvey [7] and archived residual sera that were positive for ANA, EBV IgM and Syphilis (10 sera for each) where tested using the MTTT. Any positive results were sent to the NML for immunoblot testing.

**Patient Consent Statement:** The activities described in this manuscript were conducted in fulfillment of ongoing verification of Lyme diagnostic assays used in Nova Scotia and considered a quality assurance initiative by the Nova Scotia Health Research Ethics Board which did not require full review or patient consent. All clinical specimens tested were obtained from residual samples collected for routine diagnostic testing for Lyme serology, and all data related to clinical specimens were de-identified and used solely with the intent to evaluate the performance of the MTTT algorithm.

**Results:**

Between March and July 2020, LD serology was performed on 2196 specimens using the Zeus C10/VlsE EIA, producing 1955 negative results (89%) and 241 positive or equivocal results (11%) (Fig. 1). Most of the patients in the study were from the south shore region of Nova Scotia, which has the highest incidence of LD in Canada [1].
Of the 241 positive or equivocal samples, 197 had Zeus WCS EIA completed, of which 142 were positive. Of the 55 that were negative by MTTT only 1 had a positive IgG IB (positive bands: p25, 30, 41, 58, 66 and VlsE) suggesting the MTTT was falsely negative. From the 142 LD positive patients, physicians could be contacted for clinical information on 86 patients. Of those charts unavailable for review, 38 out of 56 were IgG IB positive, suggesting a past infection.

Of the 86 charts available for review, 78 patients had clinical evidence of LD including 22 who had manifestations of early localized or early disseminated infection but did not have a positive immunoblot (either IgM or IgG, including three patients where the IgM was not tested) suggesting the MTTT detected 28% more cases of early infection compared to the STTT. The 8 remaining patients did not have a clinical syndrome compatible with Lyme Disease and were considered false positive MTTT results.

Given only 8 of 2196 patient tests were false positive, the specificity of the MTTT is estimated to be 99.6% (99.2%-99.8%), which is similar to the 99.2% specificity of the STTT previously described [3]. Of the 27 patients with early localized infection only 3 patients did not have a rash and presented with fever, myalgias, fatigue and were diagnoses and treated as LD by the local clinician. Although this ILI presentation is more nonspecific, if they are included as false positives, the specificity remains comparable at 99.4% (98.9 - 99.7).

The 60 previously healthy individuals, 10, sero positive for EBV IgM and ANA were all negative when tested using the MTTT. Of the 10 syphilis sera tested, 2 were positive on MTTT. When tested by STTT one had a positive IgM immunoblot (positive OspC, p39 and p41 bands) and a negative IgG. The other was negative for both IgM and IgG IBs.

Discussion:

The MTTT is now recognized as an acceptable alternative to the STTT in both Canada and the United States [8]. However, laboratories must validate the assay in their populations to ensure its performance is consistent with that reported in the literature. We previously demonstrated that a MTTT using a WCS EIA followed by C6 peptide EIA identified 25% more early LD cases than STTT, with a specificity of 99.56% [6]. However, with the discontinuation of the C6 EIA, we were required to revalidate the MTTT
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using an alternative. The current study shows that the MTTT using the Zeus C10/VlsE EIA followed by
the Zeus WCS EIA detected 28% more early infections while maintaining a specificity of 99.6% which is
consistent with our previous findings and those of other researchers using a variety of other EIA
combinations [3,8]. The MTTT is not without its limitations. Like the STTT, the MTTT cannot
distinguish active and past infections and cannot diagnose re-infection as antibody responses can persist
for years [9]. In our specificity panel we found 2/10 sera with positive syphilis serology were positive by
MTTT, one of which had a positive IgM IB. There were no clinical data available on these specimens to
determine if the strong reaction on the IgM WB was a true early infection or a false positive result. False
positive MTTT and STTT in sera containing syphilis antibodies have been documented in previous
studies [10,11]. Because of the possibility of false positives generated by the IgM component of the
assay, STTT can still be helpful in late infections, such as Lyme arthritis, or when false positive results
are suspected in cases where the serologic result does not fit the clinical picture [12]. Because the charts
of patients with a negative first step EIAs in this report were not reviewed, the sensitivity of the MTTT
cannot be accurately determined. Although the MTTT has improved sensitivity, literature suggests that it
is less than 70% in early localized infections. As such, patients presenting with EM should still receive
empiric treatment rather than relying on serologic results [6,8]

There are several limitations to this study. LD cases were not evaluated by the authors and categorization
of LD relied on the clinical description provided by the attending clinician using a retrospective chart
review. However, these are experienced clinicians as the majority of the cases were in a region with the
highest incident of LD in Canada. The ability to confirm early localized infection with culture or PCR
would have been informative in determining sensitivity and less susceptible to selection bias but these
techniques were not available. The ILI criteria used for categorization for early localized infection is non-
specific and could be due to other infections, however, only 3/27 cases of early localized infection
presented with ILI and if these were considered false positives, the specificity of the MTTT was
essentially unchanged. In this study several tests were ordered from emergency departments where
follow-up with the ordering clinician was difficult. We were unable to review the clinical information on
55 patients, which could have resulted in an over-representation of specificity. Although 38 patients had positive IgG WBs confirming they had been exposed to *B. burgdorferi*, we could not assign 17 patients as true positives or false positives. If all of these are considered false positives, the specificity of the MTTT would drop to 98.7% (98.14 -99.18), but it is still within the range suggested in the review by Waddell and colleagues [3] 99.2% (98.3% - 99.6%). Although, strain diversity can affect the performance of diagnostic tests it is more directly related to Western blots [13]. How it impacts the performance of the MTTT has not been assessed. However, it is possible that potentially unique strains of *B. burgdorferi* could circulate in Nova Scotia limiting its performance in other regions. Therefore, further validations studies will be required to verify the MTTT’s sensitivity for detecting early LD in other locations.

**Conclusion:**

A MTTT using the Zeus C10/VlsE followed by the Zeus WCS EIA improves the sensitivity for detection of early LD and has equivalent specificity compared to STTT. Since implementation at the Nova Scotia Diagnostic Microbiology Laboratory on April 1, 2021 the MTTT has resulted in shorter turnaround times, expedited management of patients, and cost-savings.

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**Potential Conflicts of Interest:** We report no conflicts of interest.

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References:


Figure 1. Flowchart of serological testing and chart review results. EIA: Enzyme immunoassay, IB:

1. Immunoblot, LD: Lyme Disease, PTLD: Post-treatment Lyme Disease. *15 IgM IB pos / IgG IB neg; 12
2. IgM IB neg (1 NT) / IgG IB neg. ** 38 IgG IB neg (8 IgM IB neg; 28 IgM IB pos; 2 IgM IB NT) / 7 IgG
3. IB pos. *** Of the 8 false positives All IgG IB neg; (2 IgM IB pos / 6 IgM IB NT/neg). 5 did not have
4. repeat serology, three had > 4 weeks follow-up and all remained IgG IB neg. £ Of the 27 patients with
5. early localized infection 23 (85% has EM rash); 1 had a rash that was describes as oval but 5 cm in
6. diameter; only 3 patients did not have a rash and presented with fever, myalgias, fatigue and were
7. diagnoses and treated as LD by the clinician. γ Of the 45 patients with early disseminated infection 37
8. had multiple EM; 3 had Bell’s palsy; 2 had symptoms to suggest meningitis; 1 had heart block; the
9. remaining 2 were diagnosed clinically with LD by the clinician and treated with doxycycline (1 had
10. migratory arthralgia and a borderline IgG WB; 1 had fever, arthralgia and other neurologic symptoms not
11. defined).

[^1]: 2196 specimens tested by C10/Vi/Ab
[^2]: 241 C10/Vi/Ab EIA Positive
[^3]: 1955 C10/Vi/Ab EIA Negative
[^4]: 187 Tested by WC5 EIA
[^5]: 44 Not tested using WC5
[^6]: 142 MTIT Positive
[^7]: 55 MTIT Negative
[^8]: 3 of 55 was IgG WB Positive
[^9]: Patients reviewed
[^10]: Patients unable to review
[^11]: 38 IgG WB Positive (true positive)
[^12]: 18 IgG WB Negative (no data)
[^13]: 78 LD cases (true positives)
[^14]: 8 with clinical LD (false positives)
[^15]: 27* Early localized infection
[^16]: 45** Early disseminated infection
[^17]: 0 Late LD
[^18]: 6 Past infection/PTLD

Figure 1
35x33 mm (.48 x DPI)