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# Higher C6 enzyme immunoassay index values correlate with a diagnosis of noncutaneous Lyme disease☆

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ABSTRACT

The correlation between the Food and Drug Administration–cleared C6 enzyme immunoassay (EIA) C6 index values and a diagnosis of Lyme disease has not been examined. We used pooled patient-level data from 5 studies of adults and children with Lyme disease and control subjects who were tested with the C6 EIA. We constructed a receiver operating characteristic curve using regression clustered by study and measured the area under the curve (AUC) to examine the accuracy of the C6 index values in differentiating between patients with noncutaneous Lyme disease and control subjects. In the 4821 included patients, the C6 index value had excellent ability to distinguish between patients with noncutaneous Lyme disease and control subjects [AUC 0.99; 95% confidence interval (CI) 0.99–1.00]. An index value cut point of ≥3.0 had a sensitivity of 90.9% (95% CI, 87.8–93.3) and specificity of 99.0% (95% CI, 98.6–99.2%) for Lyme disease.



## 1. Introduction

Conventional 2-tiered serologic testing for Lyme disease starts with a sensitive first-tier enzyme immunoassay (EIA). If the first-tier test is reactive (positive or equivocal), the more specific second-tier supplemental immunoblots are performed (Centers for Disease Control and Prevention (CDC), 1995). In both adults and children, the United States Food and Drug Administration (FDA)–cleared C6 EIA first tier-test is comparably sensitive to a whole cell sonicate (WCS) EIA alone but has higher specificity (Branda et al., 2011; Wormser, Schriefer, et al., 2013; Molins et al., 2014; Lipsett et al., 2016). However, as a qualitative test, the C6 EIA alone is less specific than conventional 2-tiered testing with immunoblots, and therefore, its use as a stand-alone test has not been recommended (Branda et al., 2018).

Although EIAs typically produce continuous variable optical density index values, many clinical laboratories report a categorical interpretation of this result (positive, negative, or equivocal) that is used to determine whether a second-tier serologic test will be performed. Previous work has demonstrated that WCS or VlsE EIA index values can be used

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to determine the likelihood of Lyme disease (Lipsett et al., 2015; Zwerink et al., 2018). In 1 study of children from a Lyme disease–endemic area who were being evaluated for potential Lyme disease, a WCS EIA index value ≥3.0 had a positive predictive value for Lyme disease of 99.4% [95% confidence interval (CI) 98.1–99.8%] (Lipsett et al., 2015). However, the test characteristics of the C6 EIA index value for the diagnosis of Lyme disease have not been examined.

To this end, we aggregated patient-level data from several published studies of children and adults undergoing evaluation for Lyme disease, as well as healthy control subjects. We selected studies in which participants were tested with the C6 EIA and, if reactive, supplemental IgM and IgG immunoblots were performed. Our primary aim was to examine whether the C6 EIA index value could assist clinical decision making for patients with potential Lyme disease while awaiting confirmatory immunoblot results by examining the correlation between higher C6 EIA optical density index values and a diagnosis of noncutaneous Lyme disease. Our secondary aims were to determine the correlation between higher C6 EIA optical density index values and the diagnosis of Lyme disease with any manifestation (cutaneous or noncutaneous) as well as the correlation with a positive supplemental immunoblot.

## 2. Materials and methods

### 2.1. Study design

We performed a systematic review and reanalysis of published studies to evaluate the performance of the C6 EIA index value. To this end, we aggregated patient-level data from studies of the C6 EIA test for the diagnosis of Lyme disease (Branda et al., 2011; Wormser, Schriefer, et al., 2013; Molins et al., 2014; Lipsett et al., 2016; Nigrovic et al., 2017). Each individual study was approved by the institutional review board (IRB) of the participating institution. The Boston Children's Hospital IRB deemed this study protocol exempt from additional review.

### 2.2. Study selection

We searched PubMed, Embase, and Web of Science to identify studies evaluating the performance of the commercially available C6 EIA in the diagnosis of Lyme disease (C6 *Borrelia burgdorferi* EIA, Immunetics™; Boston, MA). We limited our analysis to studies of U.S. patients of any age published prior to December 31, 2017, in which quantitative C6 index values were generated (Branda et al., 2011, 2013; Lipsett et al., 2016; Molins et al., 2014, 2015; Wormser, Schriefer, et al., 2013). We contacted corresponding authors to obtain patient-level data. We excluded 1 eligible study because data were not conveniently available for secondary analysis (Wormser, Schriefer, et al., 2013). We assessed the possibility of publication bias with visual assessment of funnel plot. For all included studies, Lyme disease cases were confirmed by the presence of objective clinical findings, appropriate epidemiologic risk, and often with a positive *B. burgdorferi* culture or PCR result from relevant tissue or fluid samples, and/or with a positive result using 2-tiered serologic testing.

One of the pediatric studies reported results of Lyme disease tests by sample rather than by patient (Lipsett et al., 2016), raising the possibility that a few children could have been included more than once in our analysis if individuals were tested multiple times over the study period. In addition to the published results, we also included unpublished pediatric data provided by this study's principal investigator (LEN): additional C6 EIA results generated using 690 normally discarded serum samples from a single center as well as 1540 samples from subjects prospectively enrolled in the ongoing Pedi Lyme Net cohort (Nigrovic et al., 2017 http://www.childrenshospital.org/research/centers-departmental-programs/pedi-lyme-net).

### 2.3. Laboratory testing for Lyme disease

In all cases, 2-tiered serology consisted of a C6 EIA followed by a supplemental immunoblot for samples with a positive or equivocal C6 EIA. All the C6 EIA results were obtained using the same commercially available diagnostic test kit. As recommended by the manufacturer, the testing laboratory converted Lyme C6 EIA optical density index values to "index values" by dividing by a standardized factor. We used cut points recommended by the assay manufacturer to classify C6 EIA index values as negative (optical density values <0.90), equivocal (optical density index values 0.90–1.09), or positive (optical density index values ≥1.10) (Immunetics websites, 2015). IgG and IgM *B. burgdorferi* immunoblots were each interpreted using standard criteria (Centers for Disease Control and Prevention (CDC), 1995) by the clinical or research laboratory performing the test. Because patients with Lyme disease who have been symptomatic for more than a month should have a *B. burgdorferi* IgG antibody response (Lantos et al., 2016; Seriburi et al., 2012), we classified patients as seronegative if they had a positive IgM immunoblot alone and had more than 30 days of symptoms (Centers for Disease Control and Prevention (CDC), 1995).

### 2.4. Data collection

We abstracted the following from the published manuscripts: age range of patients and total number of patients tested. We then contacted the corresponding authors to request the following patient-level data: nature and duration of clinical symptoms, and C6 EIA quantitative optical density index value along with IgM and IgG immunoblot results (when performed). We excluded test results obtained from patients without available data on duration of clinical signs and symptoms.

### 2.5. Lyme disease diagnosis

We defined a case of Lyme disease with either an EM skin lesion or positive 2-tiered serology in a patient with compatible symptoms (Nigrovic et al., 2017). Reviewed studies defined EM as erythematous skin lesion measuring at least 5 cm in diameter that expands over a period of days to weeks to form a large round lesion, often with partial central clearing. Extracutaneous Lyme disease was divided into the following stages: early disseminated (e.g., cranial neuritis, meningitis, carditis) and late (arthritis). Symptomatic patients without any cutaneous manifestations and negative 2-tiered Lyme disease serology as well as all asymptomatic control patients regardless of 2-tiered results were classified as not having Lyme disease.

### 2.6. Statistical analysis

Our primary goal was to examine the ability of the C6 EIA index value to predict a diagnosis of noncutaneous Lyme disease. For this analysis, we excluded patients with a diagnosis of single or multiple EM since this is the only manifestation of Lyme disease that can be diagnosed solely using clinical criteria without the need for diagnostic test results. Our secondary goals were to examine the ability of a C6 EIA index value to predict 1) a clinical diagnosis of Lyme disease, regardless of the manifestations (cutaneous or non-cutaneous), and 2) a positive supplemental Lyme disease immunoblot for patients who had an immunoblot performed.

To this end, we plotted the true-positive rate (sensitivity) vs. the false-positive rate (1 − specificity) on a receiver operating characteristic (ROC) curve using regression with clustering by study to adjust for differences. We used the area under the curve (AUC) to measure the C6 EIA index value's ability to distinguish between samples obtained from individuals from each of the selected groups. We interpreted the AUC using published standards for diagnostic accuracy: AUCs <0.7, poor discriminatory value; AUCs 0.7–0.8, minimally accurate; AUCs

0.8–0.9, good accuracy; or AUCs >0.9, excellent accuracy (Bonsu and Harper, 2003).

For patients with noncutaneous manifestations of Lyme disease, we selected the optimal C6 EIA index value cut point for discriminating between patients with and without Lyme disease. We selected the C6 index value associated with the point where the ROC "curve turns the corner," at which every incremental gain in sensitivity results in a substantial loss of specificity rounded to the closest integer for ease of application. For comparison, we examined the performance characteristics (sensitivity, specificity) of the dichotomized C6 index value for the diagnosis of Lyme disease across a range of C6 cut points for all patients. Last, we repeated the ROC curve analysis for 2 a priori selected subgroups: 1) all patients regardless of clinical presentation and 2) all patients who had an immunoblot performed.

We utilized SPSS version 23.0 for all statistical analyses (IBM SPSS Software; Armonk, NY).

## 3. Results

We included C6 EIA results from 4 published studies of well-characterized patients with Lyme disease and control subjects (Branda et al., 2011; Lipsett et al., 2016; Molins et al., 2014, 2015), plus additional unpublished data (Nigrovic et al., 2017), for a total of 5135 C6 EIA test results (Table 1). Using the funnel plot, we did not detect substantial publication bias (results not shown). Of the included C6 EIA results, 1994 (38.8%) were obtained from asymptomatic control subjects. Of the eligible C6 assay results, 589 (11.5%) were positive with an index value ≥1.10, and 69 (1.3%) were equivocal with index values between 0.90 and 1.09.

Of the 5135 included samples, 754 (14.7%) were obtained from patients with Lyme disease. Among these patients, 315 (41.8%) had cutaneous Lyme disease and 439 (58.2%) noncutaneous Lyme disease; 148 of those with noncutaneous disease had manifestations of early dissemination, and 291 had late Lyme disease. Of the 439 patients with noncutaneous Lyme disease, all had a positive or equivocal C6 EIA followed by a positive supplemental immunoblot: 187 (42.6%) were positive by IgG immunoblot alone, 159 (36.2%) were positive by both IgG and IgM immunoblots, and 93 (21.2%) were positive by IgM immunoblot alone with fewer than 30 days of symptoms.

For the 4820 patients without cutaneous Lyme disease, we used ROC regression analysis with clustering by center to examine the predictive ability of the C6 EIA index value for noncutaneous Lyme disease (Fig. 1). After adjustment, the AUC was 0.99 (95% CI 0.99–1.00), indicating "excellent" ability to discriminate between those with and without Lyme disease. We then examined the sensitivity and specificity of the C6 EIA index value for noncutaneous Lyme disease using various cut points (Table 2). Using this analysis and the ROC curve, we selected a C6 index value of ≥3.0 as the optimal cut point to identify patients who are highly likely to have non-cutaneous Lyme disease (sensitivity 92.7%, 95% CI 87.1–96.0%; specificity 99.0%, 95% CI 98.6–99.2%). For the 1014 patients with symptoms compatible with early-disseminated Lyme disease, a C6 EIA index value of ≥3.0 had sensitivity of 85.8% (95% CI 79.3–90.5%) and a specificity of 98.9% (95% CI 97.9–99.4%), and of the 1390 with symptoms compatible with late Lyme disease, a C6 EIA index value ≥3.0 had a sensitivity of 93.5% (95% CI 90.0–95.8%) and a specificity of 99.1 (98.3–99.5%) for Lyme disease.

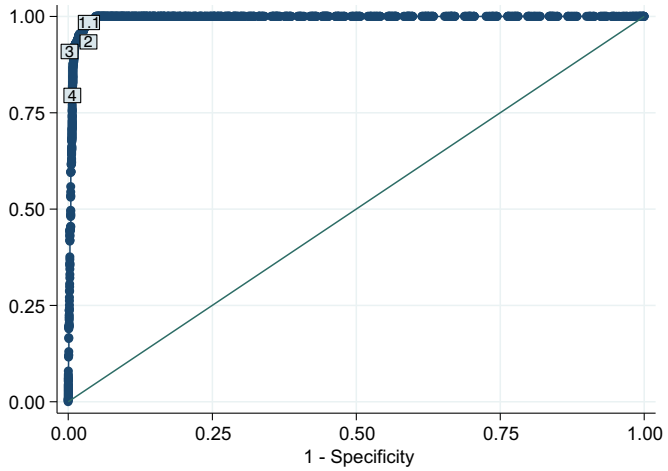


**Fig. 1.** The ROC for C6 EIA index value for Lyme disease after exclusion of cutaneous Lyme disease cases with specific C6 index value cut points indicated (boxes).

Next, we examined the ability of the C6 index value to predict all cases of Lyme disease (cutaneous and noncutaneous disease) (Fig. 2; AUC of 0.92, 95% CI, 0.86–0.96). Finally, in the 1628 patients who had an immunoblot performed, we examined the ability of the C6 index value to distinguish between patients with a positive and negative immunoblot (AUC of 0.92, 95% CI 0.86–0.94) regardless of clinical symptoms. In these adjusted secondary analyses, the C6 index value demonstrated an "excellent" ability to discriminate between 1) patients with and without Lyme disease and 2) patients with and without a positive immunoblot.

## 4. Discussion

In our systematic review of both published and unpublished patient-level data abstracted from 5 studies, there was a strong positive correlation between C6 EIA index values and a confirmed diagnosis of noncutaneous Lyme disease. The C6 index value has the potential to inform clinical decision making, as higher values are associated with a higher probability of true disease. In particular, if a patient presents with signs compatible with noncutaneous Lyme disease and the C6 EIA index value is high (≥3.0), clinicians could more confidently make targeted therapeutic decisions while awaiting supplemental immunoblot results.

Previous investigations have examined the C6 EIA as a dichotomous test: nonreactive (negative) versus reactive (positive or equivocal). Although the sensitivity of the C6 test alone is high, compared with standard 2-tiered testing, the specificity of the dichotomous test result

**Table 1**
Included studies.

| First author | *Journal (year)* | Overall $N = 5135$ | Patient ages | Noncutaneous Lyme $N = 439$ | Cutaneous Lyme $N = 315$ | Control subjects $N = 4381$ |
|---|---|---|---|---|---|---|
| Branda et al., 2011 | *Clin Infect Dis* (2011) | 1391 | Adults | 28 | 63 | Asymptomatic ($n = 1300$) |
| Lipsett et al., 2016 | *Clin Infect Dis* (2015) | 1634[a] | Children[b] | 149 | 21 | Symptomatic ($n = 1277$)<br>Asymptomatic ($n = 187$) |
| Molins et al., 2014 | *Clin Infect Dis* (2015) | 139 | Adults | 0 | 139 | n/a |
| Molins et al., 2015 | *J Clin Microbiol* (2016) | 431 | Adults | 30 | 54 | Symptomatic ($n = 144$)<br>Asymptomatic ($n = 203$) |
| Nigrovic et al., 2017 | *Pedi Lyme Net* (Unpublished) | 1540 | Children[b] | 232 | 38 | Symptomatic ($n = 966$)<br>Asymptomatic ($n = 304$) |

[a] Includes C6 assay results from 690 pediatric samples not included in the published manuscript.
[b] All patients ≤21 years of age.

**Table 2**
Performance of C6 EIA index value by cut point to predict noncutaneous Lyme disease.

| C6 EIA index value cut point | No. with Lyme disease ($N = 439$) n | No. without Lyme disease ($N = 4381$) n | Sensitivity % (95% CI) | Specificity % (95% CI) |
|---|---|---|---|---|
| ≥0.90 | 439 | 219 | 100% (99.1–100%) | 95.0% (94.3–95.6%) |
| ≥1.10 | 432 | 157 | 98.4% (96.8–99.2) | 96.4% (95.8–96.9) |
| ≥2.0 | 410 | 67 | 93.4% (90.7–95.4) | 98.5% (98.1–98.8) |
| ≥3.0 | 399 | 45 | 90.9% (87.8–93.3) | 99.0% (98.6–99.2) |
| ≥4.0 | 349 | 31 | 79.3% (75.5–83.9) | 99.3% (99.0–99.5) |
| ≥5.0 | 270 | 23 | 61.5% (56.9–65.9) | 99.5% (99.2–99.7) |

alone is not high enough to definitely establish the diagnosis of Lyme disease without supplemental immunoblot results (Branda et al., 2011; Lipsett et al., 2016; Molins et al., 2014; Wormser, Schriefer, et al., 2013). Because the 2 steps in this diagnostic algorithm are usually performed sequentially and most clinical laboratories outsource the immunoblotting step to regional laboratories (Forrester et al., 2015; Wormser, Levin, et al., 2013), the immunoblot results may be delayed for several days and are usually not available when initial management decisions must be made (Cohn et al., 2012; Deanehan et al., 2013). In contrast, the first-tier test (whether the C6 EIA or another EIA) is often performed on-site with shorter turnaround time. Our investigation is the first to demonstrate a correlation between higher C6 index values and a diagnosis of Lyme disease, and between higher C6 EIA index values and a positive supplemental immunoblot. Based on our results, we suggest that the C6 EIA index value has actionable diagnostic value over and above the qualitative interpretation (reactive versus nonreactive), which could be factored among other clinical and laboratory findings in the initial assessment and management of patients with potential noncutaneous Lyme disease. In some instances, this might help avoid unnecessary invasive procedures to investigate alternative diagnoses or initiation of unnecessary and potentially harmful medical therapies (Dart et al., 2018).

For example, in patients presenting with acute monoarticular arthritis, the clinician must promptly make a judgment about whether to perform arthrocentesis or operative joint washout to evaluate for a septic arthritis (Dart et al., 2018; Deanehan et al., 2013). Although Lyme arthritis might be suspected, the clinical overlap between Lyme and septic arthritis can make challenging the decision to forgo these invasive diagnostic procedures and provide only targeted antimicrobial therapy for *B. burgdorferi* infection without laboratory confirmation. Similarly, in patients presenting with unilateral peripheral facial nerve palsy, a judgment must be made whether to provide corticosteroids to treat Bell's palsy, whether to provide antimicrobial therapy for acute Lyme neuroborreliosis, or both (Garro and Nigrovic, 2018; Jowett et al., 2017). In such cases, a higher C6 EIA index value could provide strong rationale to target initial therapy for Lyme disease.

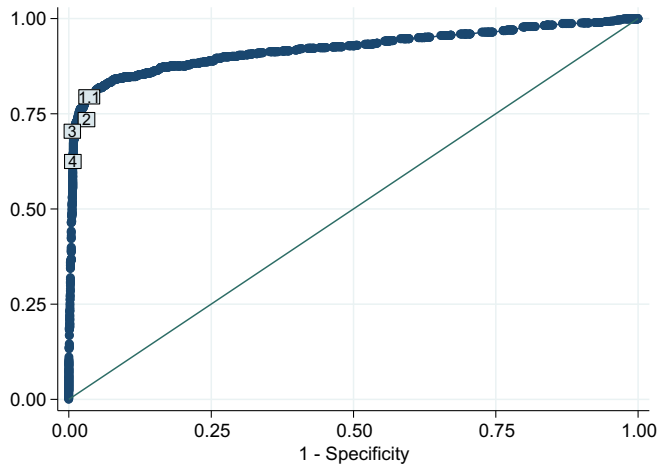


**Fig. 2.** The ROC for C6 EIA index value for all Lyme disease cases with specific C6 index value cut points indicated.

We were unable to identify a C6 index value cut point with sufficient specificity to obviate the need for a supplemental immunoblot while maintaining sufficient sensitivity to be clinically relevant. Given the large volume of annual Lyme disease testing (approximately 3.4 million serologic tests for Lyme disease were performed in 2008 on 2.4 million unique patients) (Hinckley et al., 2014), the slightly lower specificity of a single-tier C6 test would substantially increase the number of false-positive test results, leading to Lyme disease overdiagnosis. Therefore, for patients with potential noncutaneous Lyme disease, we suggest that the supplemental immunoblot should still be obtained even for patients with high C6 EIA index values to complete the evaluation.

Clinical laboratories offer interpretations of study results in order to assist clinical decision making. Our findings support the following suggestions when reporting C6 EIA index values: For C6 index values <0.9, no further Lyme disease testing would be suggested for the submitted sample. However, as Lyme disease serology may be falsely negative in early Lyme disease, clinicians should consider repeating the test after several weeks if the clinical suspicion of early Lyme disease remains high (Kaiser, 2000; Marques, 2015). For all patients with a C6 index value ≥0.9, the clinician should obtain a supplemental immunoblot. A C6 index value ≥3.0, in the appropriate clinical context, supports a presumptive diagnosis of Lyme disease while awaiting supplemental immunoblot results. The current FDA-cleared C6 EIA assay is a moderate-complexity test performed by appropriately trained laboratory technicians with results available in as few as a couple of hours. Future point-of-care C6 or other new-generation first-tier assays may be able to provide results within an even more clinically relevant time frame. Ideally, these assays should provide the quantitative index value to the treating clinician to most effectively assist initial clinical decision making.

Our study should be interpreted in the context of its limitations. First, our analysis was limited to published evaluations of the C6 EIA assay, although we did not detect any substantial publication bias. Secondly, we only used studies utilizing serum primarily collected from patients in the Northeast of the U.S., and findings may not be applicable to other regions where *Borrelia* species may differ (Krause et al., 2018), although previous work has suggested that the C6 EIA test may perform similarly for *B. burgdorferi* infections acquired in Europe (Branda et al., 2013; Wormser et al., 2014). Third, we had only a single C6 EIA index value per tested sample, and we cannot comment on the test–retest reliability of the diagnostic assay. Fourth, we did not evaluate

study patients for other emerging *Borrelia* infections (e.g., *B. miyamotoi*), and the C6 EIA may also be positive in serum samples collected from patients with these infections (Molloy et al., 2018). Fifth, the included Lyme disease diagnostic tests were performed in a variety of clinical and research laboratories. However, this mimics the real-world situation of clinical Lyme disease testing. Importantly, all studies utilized the same FDA-cleared C6 EIA diagnostic kit, and all supplemental immunoblots were performed and interpreted in 1 of 3 large commercial laboratories using standardized interpretative criteria (Centers for Disease Control and Prevention (CDC), 1995). Sixth, standard 2-tier serologic testing has well-recognized limitations that include both false positives and negatives. Although we did not knowingly include patients with previous Lyme disease, a *B. burgdorferi* antibody response can persist for years or even decades even following effective antimicrobial therapy (Kalish et al., 2001). For our analysis, we assumed that patients with positive 2-tiered serology and compatible symptoms had active Lyme disease. As we included only a single Lyme disease test at a single point in time, some study patients with early Lyme disease may have had falsely negative 2-tiered serology. In practice, clinicians should consider repeating Lyme disease testing after a few weeks for patients with high clinical suspicion for Lyme disease and a negative initial Lyme disease test (Kaiser, 2000). Lastly, we did not compare the performance of the C6 EIA index value to that of other first-tier EIA tests (e.g., WCS EIA) (Lipsett et al., 2015), so we cannot comment on the relative accuracy.

## 5. Conclusions

C6 EIA index values can provide valuable, actionable information to guide initial clinician decision making for children or adults with possible Lyme disease. Clinical laboratories should consider reporting C6 EIA index values, along with annotations to aid interpretation and suggest a course of action to the treating clinician. The C6 EIA index value could be factored in along with clinical signs and epidemiological risk factors and, in some cases, could justify targeted initial therapy while avoiding potentially harmful interventions directed toward other entities before the results of supplemental immunoblots become available. Future prospective studies should measure the impact of providing clinicians quantitative index values with interpretation on the care of patients being evaluated for Lyme disease.

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