



Evaluation of the Modified Two-Tiered Testing Method for Diagnosis of Lyme Disease in Children

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ABSTRACT Conventional two-tiered testing (CTTT) for Lyme disease includes a first-tier enzyme immunoassay (EIA) followed by a supplemental immunoblot, and modified two-tiered testing (MTTT) relies on two different sequential EIAs without the inclusion of an immunoblot. MTTT has shown promising results as an alternative strategy for the diagnosis of Lyme disease in adults but has not yet been evaluated in children. We performed a cross-sectional study of children and adolescents ≤ 21 years of age undergoing clinical investigation for suspected Lyme disease. Serum specimens were analyzed with both a whole-cell sonicate (WCS) and a C6 EIA, with a supplemental immunoblot if either EIA was positive or equivocal. We compared CTTT (WCS EIA followed by supplemental immunoblot) to MTTT (WCS EIA followed by C6 EIA) using McNemar's test to evaluate for agreement beyond chance alone. We then used a kappa statistic to measure level of the agreement between testing strategies. We included 1,066 serum specimens, of which 156 (14.6%) had a positive CTTT and 165 (15.5%) had a positive MTTT. There were no significant differences between MTTT and CTTT ($P = 0.16$). Although the overall agreement between MTTT and CTTT was high (kappa, 0.88; 95% confidence interval, 0.84 to 0.92), 33 children had discordant test results. In a cohort of children and adolescents undergoing investigation for suspected Lyme disease, CTTT and MTTT results agreed in most cases. Since immunoblots are time-consuming, laborious, and challenging to interpret, MTTT provides a promising alternate Lyme disease testing strategy for children.

KEYWORDS *Borrelia burgdorferi*, Lyme disease, enzyme immunoassay, pediatrics, serologic testing

Conventional two-tiered serologic testing for Lyme disease includes a sensitive first-tier enzyme immunoassay (EIA) followed by a more specific supplemental immunoblot if the first-tier test is positive or equivocal (1). However, immunoblots are more expensive than EIAs, are subject to variability in interpretation, and are almost exclusively run by regional commercial laboratories that take several days to return results (2). To circumvent these challenges, Lyme disease testing strategies that obviate an immunoblot have been suggested.

A modified two-tiered testing (MTTT) strategy using a whole-cell sonicate (WCS) EIA followed by a C6 peptide EIA has been proposed (3, 4). In adults, this testing strategy has demonstrated higher sensitivity in early Lyme disease, and similar specificity overall, compared to CTTT (3, 4). MTTT has not yet been systematically evaluated in children. Given the potential for a more rapid and economical testing strategy, our goal was to compare MTTT with CTTT in a group of children and adolescents undergoing clinical investigation for suspected Lyme disease.

MATERIALS AND METHODS

Study design and setting. We performed a cross-sectional study using excess serum specimens from children presenting to the Boston Children's Hospital (i.e., outpatient clinic, emergency department,

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or inpatient wards) between June 2014 and October 2017, who had Lyme disease testing ordered by their treating clinician. The study protocol was approved by the Boston Children's Hospital Institutional Review Board with waiver of informed consent.

Subject identification. We included serum specimens collected from children and adolescents ≤ 21 years of age who had clinical symptoms compatible with early Lyme disease (a single erythema migrans [EM] lesion), early disseminated Lyme disease (multiple EM lesions, lymphocytic meningitis, carditis, and/or facial nerve palsy), or late Lyme disease (arthritis) and who were tested for Lyme disease using the conventional two-tiered testing approach. We excluded specimens collected from children with only nonspecific constitutional symptoms such as fatigue or fever and specimens obtained from children without an available clinical history. We included multiple specimens from the same child if obtained at least 30 days apart.

Data collection. We abstracted the following patient-related data from the medical record onto a structured data form using Redcap (5): demographics (age, sex), clinical symptoms, duration of symptoms, and results of serologic testing for Lyme disease (WCS EIA and, if performed, immunoblotting). We classified each serum specimen by stage using the clinical histories abstracted by the research team.

Clinical testing (CTTT). When performed for clinical diagnostic purposes, serologic testing for Lyme disease was performed at a single commercial reference laboratory (ARUP National Laboratories, Salt Lake, UT) using the MarDx Lyme EIA and Lyme Western blot kits (Trinity Biotech, Tray, Ireland). As recommended by the manufacturer, the laboratory converted Lyme EIA optical density values to "index values" by dividing the optical density value by a standardized factor. Results were interpreted according to standard cut points provided by the manufacturer: ≥ 1.20 (positive), 1.00 to 1.19 (equivocal), and < 1.00 (negative). All specimens with equivocal or positive Lyme EIA index values were reflexively evaluated using both immunoglobulin G (IgG) and immunoglobulin M (IgM) immunoblots. Immunoblots were classified as positive or negative according to CDC criteria (1).

Research testing (MTTT). Excess serum specimens were stored at -80°C after collection and sent in batches to the research laboratory of John Branda (Massachusetts General Hospital; Boston, MA), where the commercially available C6 Lyme EIA (Oxford Immunotec USA; Immunetics, Inc., Marlborough, MA) was performed according to manufacturer's instructions. We interpreted the resulting index values according to standard cut points provided by the manufacturer: ≥ 1.10 (positive), 0.91 to 1.09 (equivocal), and ≤ 0.90 (negative). We classified both positive and equivocal tests as reactive in our analysis. Children with a positive or equivocal C6 EIA who did not have a clinical immunoblot had research immunoblot analyses performed at the same commercial laboratory (ARUP Laboratories, Salt Lake, UT).

Lyme disease two-tier serology. We defined the overall CTTT result as seropositive if the WCS EIA was positive or equivocal (index value ≥ 1.0) and the supplemental immunoblot step was positive (1). The immunoblot step was classified as positive if either the IgG immunoblot was positive according to standardized criteria or if the IgM immunoblot was positive by standardized criteria in a patient with a symptom duration of ≤ 30 days (1, 6–8). We defined the overall MTTT result as seropositive if the WCS EIA was positive or equivocal (index value ≥ 1.0) and the supplemental C6 EIA was positive or equivocal (index value ≥ 0.91), regardless of symptom duration or suspected stage of Lyme disease.

Analysis of discordant CTTT and MTTT results. For specimens with discordant CTTT and MTTT results, we reviewed clinical histories, EIA index values and immunoblot bands to further categorize children into one of four groups: partial seroconversion, partial seroreversion, false-positive MTTT, or false-negative MTTT. Specimens from children with symptoms of acute Lyme disease and with short duration of symptoms (≤ 30 days) whose serologic testing was consistent with a developing antibody response were classified as partial seroconversion. Specimens from children with a history of previous Lyme disease with evidence of a waning antibody response were classified as partial seroreversion. In some cases, MTTT was positive, but a review of the clinical history and serologic findings revealed that Lyme disease was unlikely; such cases were classified as false-positive MTTT. If MTTT was negative but medical record review and serologic findings strongly suggested active Lyme disease, the case was classified as false-negative MTTT.

Statistical analysis. First, we used the McNemar's test to evaluate for agreement between CTTT and MTTT beyond what would be expected by chance alone. Next, we measured both the percent agreement and the kappa statistic between the CTTT and MTTT results for all enrolled subjects and for subcategories defined by suspected Lyme disease stage. We classified the level of agreement based on published standards for the kappa statistic point estimate: 0.81 to 1.00 (almost perfect), 0.61 to 0.80 (substantial), 0.41 to 0.60 (moderate), 0.21 to 0.40 (fair), and 0 to 0.20 (slight) (9). All analyses were performed using SPSS version 24.0 (10).

RESULTS

Over the study period, we collected 1,581 serum specimens. After excluding 377 specimens collected from children with nonspecific symptoms and 138 specimens from children without available clinical histories, we included 1,066 serum specimens. The median patient age was 10.8 years (interquartile range, 6.6 to 15.0 years) with the following age distribution: 77 (7.2%) were ≤ 2 years old, 276 (25.9%) were 3 to 7 years old, 393 (36.9%) were 8 to 13 years old, 283 (26.5%) were 14 to 18 years old, and 37 (3.5%) 19 to 21 years old, and 473 (44% of the patients) were male. Specimens came from patients with symptoms of the following Lyme disease stages: 21 (2%) early (single EM lesion), 371 (35%) early-disseminated, and 674 (63%) late.

TABLE 1 Agreement between MTTT and CTTT strategies overall and by symptom stage

Stage (n)	MTTT result	CTTT result		% Agreement (95% CI)	Kappa (95% CI)
		Positive	Negative		
Overall (1,066)	Positive	144	21	96.9 (95.7–97.8)	0.88 (0.84–0.92)
	Negative	12	889		
Erythema migrans lesion (21)	Positive	3	1	95.2 (77.3–99.2)	0.83 (0.51–1.00)
	Negative	0	17		
Symptoms of early disseminated Lyme disease (371)	Positive	45	10	95.7 (93.1–97.3)	0.82 (0.74–0.90)
	Negative	6	310		
Symptoms of late Lyme disease (674)	Positive	96	10	97.6 (96.2–98.5)	0.91 (0.87–0.95)
	Negative	6	562		

There were no significant differences between the MTTT and CTTT strategies ($P = 0.16$). The MTTT and CTTT agreed in 96.9% of the tests (95% confidence interval [CI], 95.7 to 97.8%) with an “almost perfect” kappa value of 0.88 (95% CI, 0.84 to 0.92). When stratified by suspected stage of Lyme disease, the kappa statistic was higher in suspected late-stage disease than in suspected early and early-disseminated disease (Table 1).

The results of the two testing algorithms differed in 33/1,066 specimens (3.1%). Of the 12 specimens that were seropositive by CTTT but seronegative by MTTT (Table 2), three were from children with symptoms of early-disseminated or late Lyme disease of <30 days' duration and were consistent with a developing antibody response (i.e., partial seroconversion). Seven specimens came from children with clinical history and serologic findings, suggesting past Lyme disease with a waning antibody response at the time of index serum sample collection (partial seroreversion). The remaining two samples had been collected from patients with active early disseminated or late Lyme disease; the C6 EIA was unexpectedly negative in both cases, resulting in a negative MTTT outcomes. These cases were categorized as having false-negative MTTT results. Further detail about the subcategorization of these cases is provided in the supplemental material.

Among the 22 specimens with positive MTTT and negative CTTT (Table 3), four were from children with serological findings compatible with a blunted antibody response due to early antimicrobial administration for known past early Lyme disease (categorized as partial seroconversion). Two of these specimens were from children with

TABLE 2 Clinical and serologic information from children with positive conventional two-tiered testing and negative modified two-tiered testing ($n = 12$)^a

Suspected stage of Lyme disease	Age (yr)	Symptom duration ≤30 days	eIA		Band(s) (kDa)		Classification	Subcategory ^b
			WCS	C6	IgG	IgM		
Early disseminated	8	N	1.21	0.73	58, 45, 41, 39, 28, 23, 18	23	PSR	PSR1
	9	Y	4.45	0.42	66, 45, 41, 23, 18	41, 23	FNM	FNM1
	11	Y	1.28	0.40	None	41, 23	PSC	PSC1
	13	Y	1.19	0.22	None	41, 23	PSC	PSC1
	15	Y	1.70	0.80	93, 58, 41, 39, 18	23	PSR	PSR2
	17	Y	1.12	0.32	93, 66, 45, 41, 23	None	PSR	PSR2
Late	3	Y	6.30	0.07	93, 66, 58, 45, 41, 39, 30, 28, 23, 18	41, 39, 23	PSR	PSR4
	3	Y	1.60	0.88	93, 66, 58, 41, 39, 23, 18	39, 23	FNM	FNM2
	14	Y	1.07	0.45	66	41, 23	PSR	PSR5
	15	N	2.04	0.80	58, 41, 39, 23, 18	None	PSR	PSR1
	15	Y	1.73	0.87	66, 58, 45, 41, 39, 23, 18	None	PSR	PSR3
	16	Y	1.06	0.66	66	41, 23	PSC	PSC1

^aPSC, partial seroconversion; PSR, partial seroreversion; FNM, false-negative MTTT.^bSee the the supplemental material for category descriptions.

TABLE 3 Clinical and serologic information from children with positive modified two-tiered testing and negative conventional two-tiered testing ($n = 21$)^a

Suspected stage of Lyme disease	Age (yr)	Symptom duration ≤ 30 days	eIA		Band(s) (kDa)		Classification	Subcategory ^c
			WCS	C6	IgG	IgM		
Early	6	Y	1.99	1.35	41	None	PSC	PSC1
Early disseminated	2	Y	1.10	0.98	41	None	FPM	FPM5
	3	Y	1.96	1.49	58, 41	None	FPM	FPM5
	4	N	1.01	1.52	66, 41	None	FPM	FPM4
	9	N	1.11	1.10	41	None	FPM	FPM1
	11	Y	1.72	1.06	41	None	FPM	FPM5
	11	N	1.13	1.35	41, 23	41, 23	FPM	FPM6
	12	N	1.18	1.05	41	None	FPM	FPM1
	13	N	2.41	2.15	41, 39, 18	None	PSR	PSR6
	15	N	4.26	6.50	66, 41, 23	41, 39, 23	PSC	PSC3
16	N	1.59	5.40	None	41, 23	PSC	PSC3	
Late	2	Y	1.97	3.01	41, 39	None	FPM	FPM3
	3	Y	4.29	6.69	45, 41	None	FPM	FPM2
	5 ^b	Y	1.11	1.23	41	None	PSC	PSC2
	6	N	2.42	5.07	93, 58, 39, 18	41	PSR	PSR6
	8	N	1.06	3.12	66, 41, 23, 18	None	PSR	PSR6
	9	N	1.08	1.02	41	None	FPM	FPM4
	11	Y	4.68	1.16	66, 41	None	FPM	FPM3
	13	N	1.01	0.99	None	None	FPM	FPM4
	15	Y	1.22	1.04	None	None	FPM	FPM4
17	N	1.07	1.04	39	None	FPM	FPM4	

^aPSC, partial seroconversion; PSR, partial seroreversion; FPM, false-positive MTTT.

^bThis child had a recent history of physician-diagnosed erythema migrans.

^cSee the supplemental material for category descriptions.

symptom durations of <30 days at the time of testing. One specimen was from a child with total symptom duration of 5 weeks but who had been treated with a course of doxycycline at symptom onset for multiple EM lesions and subsequently developed meningismus and papilledema. One specimen was from a child with untreated early localized disease for whom both EIAs were positive but the immunoblot results were negative, suggesting a developing, immature antibody response. Three specimens with positive MTTT and negative CTTT results were from children in whom early or late disseminated Lyme disease had been on the clinical differential diagnosis at the time of initial presentation but who were subsequently assigned an alternative diagnosis and were not treated for Lyme disease. In each case, reactivity in Lyme serologic tests—including the presence of several specific IgG Western immunoblot bands and WCS and C6 EIA index values well above the cut points—was considered most likely reflective of undocumented past exposure with partial seroreversion. The remaining 14 specimens were from children without a previous diagnosis of Lyme disease who had immunoblots demonstrating minimal specific antibody responses and typically had low-positive EIA index values. These 14 cases were categorized as having false-positive MTTT results, although undocumented past exposure with partial seroreversion could not be ruled out. Further detail about the subcategorization of these cases is provided in the supplemental material.

DISCUSSION

In a cross-sectional study of children and adolescents undergoing clinical investigation for suspected Lyme disease, we found that MTTT and CTTT had an almost perfect agreement (9). Although test results differed in a minority of specimens, our findings support the use of MTTT as an alternate testing strategy for the diagnosis of Lyme disease.

Until recently, supplemental immunoblots were considered essential to confirm the serological diagnosis of Lyme disease. The dependence on immunoblots presents multiple challenges: technical difficulty, increased costs (more than twice as much as

EIAs) (11), and the potential for subjective interpretation. A number of studies in adults have examined the performance of MTTT, in which two different EIAs are used sequentially or concurrently, without the use of immunoblots (3, 4). MTTT has shown superior sensitivity to CTTT in early Lyme disease, owing to the lack of a mature antibody response in this earliest stage of illness (3, 4). In general, EIAs detect an anti-*B. burgdorferi* antibody response sooner after infection compared to immunoblots, because immunoblot interpretive criteria require reactivity against multiple specific antigens, and an expanded antibody response takes time to develop. MTTT has similar specificity and is more cost-effective compared to CTTT (3, 4, 11). To our knowledge, our study is the first to evaluate an MTTT algorithm for the diagnosis of Lyme disease in children.

CTTT and MTTT had the highest agreement for specimens from children with signs and symptoms of late-stage Lyme disease (arthritis). Since robust host antibody production is usually present in late-stage Lyme disease, most serologic tests from patients with late-stage disease will demonstrate reactivity. In contrast, patients with solitary EM or early-disseminated Lyme disease typically have an immature, developing antibody response (12, 13), and reactivity may be demonstrable using one test but not another. Although WCS EIA and the C6 EIA are not completely independent tests (14), these two EIAs react to different sets of antibodies, limiting the overlap of their false-positive distributions (4). The high specificity of the C6 EIA (3, 14–17) might allow this test to replace the immunoblot, without a loss in overall specificity.

Our study has several limitations. First, we limited our study to children with objective signs and symptoms compatible with acute Lyme disease. Therefore, our findings should not be applied to children with nonspecific constitutional symptoms, a clinical situation in which Lyme disease testing is not routinely recommended (12). Second, we lack a definitive gold standard for the diagnosis of Lyme disease, as the clinical features are not pathognomonic unless a classical bulls-eye skin rash is present, and Lyme disease serology can produce both false-positive and false-negative test results (18, 19). We relied on CDC criteria for interpretation of serologic testing. Thus, children with acute arthritis of <30 days' duration with a positive EIA result and positive supplemental IgM alone were considered to have positive CTTT. Patients with Lyme arthritis are generally expected to have a well-expanded antibody response (and thus a positive IgG immunoblot result) (20, 21). A polyvalent MTTT (e.g., WCS EIA followed by C6 EIA) would not distinguish between positive IgM and IgG antibody responses; alternative MTTT strategies, including IgG/IgM-based EIAs, would offer more specific information about the antibody response in these patients. However, common practice for children presenting with acute onset monoarthritis with a positive EIA and IgM but negative IgG is to treat for Lyme disease. Finally, our study was underpowered to compare test performance in early Lyme disease. Although larger studies of children with EM lesions are needed to compare testing algorithms, Lyme disease diagnosis is based on the appearance of the skin lesion.

In conclusion, we found that in a cross-sectional study of children and adolescents undergoing clinical investigation for suspected Lyme disease, CTTT (WCS EIA followed by immunoblotting) and MTTT (WCS EIA followed by C6 EIA) testing algorithms had excellent agreement, with the highest level of agreement in children with signs and symptoms of late Lyme disease. Given the well-known limitations of immunoblot-based testing, MTTT provides a promising alternative testing strategy for children and adolescents with suspected Lyme disease. New host- or pathogen-based approaches for the diagnosis of Lyme disease are still needed to avoid both under- and overdiagnosis (19, 22).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.00547-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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ADDENDUM IN PROOF

On 29 July 2019, the Federal Drug Administration cleared several EIAs (not the ones used in this study) for use in modified two-tiered testing (MTTT) protocols without the use of immunoblots (23). Shortly thereafter, the Centers for Disease Control and Prevention (CDC) issued an update to their recommendations for the serologic diagnosis of Lyme disease (24). The CDC now recommends MTTT protocols as acceptable alternatives to conventional two-tiered testing (CTTT) protocols when assays cleared by the FDA for this purpose are used. Based on this guidance and the findings of our study, clinicians and clinical laboratories may consider adopting an MTTT strategy for children with suspected Lyme disease.

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