JCM
Journals.ASM.org

Collection and Characterization of Samples for Establishment of a Serum Repository for Lyme Disease Diagnostic Test Development and Evaluation

Claudia R. Molins,[a] Christopher Sexton,[a] John W. Young,[a] Laura V. Ashton,[b] Ryan Pappert,[a] Charles B. Beard,[a] Martin E. Schriefer[a]

Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA[a]; Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado, USA[b]

Serological assays and a two-tiered test algorithm are recommended for laboratory confirmation of Lyme disease. In the United States, the sensitivity of two-tiered testing using commercially available serology-based assays is dependent on the stage of infection and ranges from 30% in the early localized disease stage to near 100% in late-stage disease. Other variables, including subjectivity in reading Western blots, compliance with two-tiered recommendations, use of different first- and second-tier test combinations, and use of different test samples, all contribute to variation in two-tiered test performance. The availability and use of sample sets from well-characterized Lyme disease patients and controls are needed to better assess the performance of existing tests and for development of improved assays. To address this need, the Centers for Disease Control and Prevention and the National Institutes of Health prospectively collected sera from patients at all stages of Lyme disease, as well as healthy donors and patients with look-alike diseases. Patients and healthy controls were recruited using strict inclusion and exclusion criteria. Samples from all included patients were retrospectively characterized by two-tiered testing. The results from two-tiered testing corroborated the need for novel and improved diagnostics, particularly for laboratory diagnosis of earlier stages of infection. Furthermore, the two-tiered results provide a baseline with samples from well-characterized patients that can be used in comparing the sensitivity and specificity of novel diagnostics. Panels of sera and accompanying clinical and laboratory testing results are now available to Lyme disease serological test users and researchers developing novel tests.

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and is transmitted to humans by the bite of infected *Ixodes* ticks. The clinical course of disease can be described as occurring in three stages: early localized, early disseminated, and late disseminated. Each stage of disease may involve multiple tissues and organ systems, including the skin, heart, nerves, and joints (1). However, beyond the presence of an erythema migrans (EM) in the context of epidemiological risk, individual signs and symptoms of disease are not sufficiently specific to make a diagnosis (1, 2).

In contrast to many bacterial infections, the recovery of *B. burgdorferi* and direct detection of agent-specific markers are not particularly useful for diagnosis. This is due primarily to the extreme sparsity of spirochetes in most infected tissues (3). Nevertheless, a natural antibody response to the pathogen develops over the early weeks of infection and persists. These characteristics of Lyme disease have motivated efforts to identify and characterize dozens of agent-specific immune-dominant antigens and epitopes (1). Consequently, serological assays measuring antibody responses and profiles have been the mainstay of laboratory confirmation (1), as well as the basis of all Food and Drug Administration (FDA)-cleared tests for Lyme disease. In 1995, the Centers for Disease Control and Prevention (CDC) recommended a two-tiered serologic testing algorithm that utilizes a first-tier enzyme immunoassay (EIA) followed by second-tier Western immunoblot (WB) assays to standardize laboratory evidence of exposure to *B. burgdorferi* (4). Despite this recommendation, variability in test performance has led to substantial confusion and uncertainty regarding the appropriate use and interpretation of test results (3, 5–7). Many of the reported performance variances may be linked to test sample heterogeneity. Additionally, the comparative evaluation of existing and new tests suffers from a lack of a well-characterized sample repository. In 2007, the CDC and the National Institutes of Health (NIH) undertook an effort to address this shortcoming by collecting sera from patients at all stages of *B. burgdorferi* infection and negative controls that included healthy individuals and patients with other diseases known to challenge the specificity of serological tests for Lyme disease. This report describes those efforts, along with the documentation and characterization of the serum samples acquired. Furthermore, the availability of samples from the Lyme serum repository (LSR) for research and the development of improved serology tests are discussed.

MATERIALS AND METHODS

Serum acquisition and acceptance. Sera from positive (Lyme disease) and negative (non-Lyme disease) controls were obtained prospectively through contracts established between the CDC and external providers. Review of contract proposals assessed the provider's case definition and proof of disease, inclusion and exclusion criteria, informed consent, clinical history questionnaire, recruitment procedures, sample handling and tracking, and budget justification. Additionally, individual provider's ex-

Received 22 May 2014 Returned for modification 15 June 2014
Accepted 8 August 2014
Published ahead of print 13 August 2014
Editor: R. Patel
Address correspondence to Martin E. Schriefer, mms7@cdc.gov.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.01409-14.
Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JCM.01409-14

perience based on their peer-reviewed publications and ability to recruit specific donors, collect samples, provide deliverables, and meet calendar milestones were evaluated. Each selected provider was required to obtain local and CDC Institution Review Board (IRB) approval prior to enrollment of patients. The final inclusion and exclusion criteria used to identify eligible and ineligible participants for each contract were agreed upon by the contract provider and the CDC. Lyme disease serology or results from two-tiered testing did not play a role in patient enrollment except for inclusion of late-stage Lyme arthritis patients. This was done to minimize reproofing of samples for early disease (i.e., providing samples for the purpose of test evaluation and improvement that had already been shown to be seropositive) and to recruit a potentially broader Lyme disease patient population. Although early Lyme disease patients could be two-tiered negative, they were required to have well-documented clinical and laboratory (PCR and/or culture) evidence of infection.

Collected sera were stored at −20°C for up to 2 weeks or at −70°C for longer periods. The sera were transferred on dry ice to the Division of Vector-Borne Diseases at the CDC in Fort Collins, CO, along with supporting clinical and laboratory data. Upon arrival, samples were given a CDC identifier number, thawed, heat inactivated at 56°C for 30 min, aliquoted (100 μl) into 0.5-ml conical tubes with screw caps, and stored at −70°C. Heat inactivation was performed to minimize exposure risk to potential pathogens in serum samples by end users of the LSR.

Sample characterization. Sera from all individuals (positive and negative controls) were tested at the CDC for antibodies *to B. burgdorferi*. Testing was performed in a blinded fashion by assigning a random number to each sample prior to testing. All serologic assays were performed following the manufacturer's instructions. The CDC-recommended two-tiered testing algorithm (4) was performed using FDA-cleared assays for Lyme disease. The assays consisted of a first-tier whole-cell sonicate (WCS) EIA (Vidas Lyme IgM and IgG Polyvalent Assay, bioMérieux, Inc., Durham, NC), followed by second-tier IgM and IgG WBs (MarDx Diagnostics, Inc., Carlsbad, CA). The immunoblotting results were interpreted according to the guidelines proposed by the CDC (4). Samples were considered two-tiered positive if the first-tier test was equivocal or positive and the second-tier IgM and/or IgG WB was positive. For the IgM blot to be positive, at least 2 of 3 Lyme-specific bands had to be scored as meeting the test intensity threshold, and for IgG blot positivity, at least 5 of 10 Lyme-specific bands had to be scored as meeting the test intensity threshold. IgM WBs were not considered in the final interpretation of two-tiered test results for serum samples collected from patients with >30 days of illness. All WBs were read by three individuals, and the interpretations were compared. If two out of three reads agreed, the read that was in agreement was accepted as final. If the reads of all three individuals disagreed, a consensus read was determined by all three readers.

Culture and PCR of samples from enrolled Lyme patients. Samples from patients with early localized (only an EM) and early disseminated (Lyme carditis or Lyme neuroborreliosis) Lyme disease were tested by culture and PCR when possible. Culture and PCR were performed according to published methods (8–11). Additionally, joint fluid from patients with late disseminated Lyme arthritis was tested by PCR using the published methods of Li et al. (12). All culture and PCR were performed by the sample providers.

Panel types/composition. Serum panels representing both Lyme disease patients and non-Lyme disease patients were formulated to meet anticipated needs of Lyme disease clinical laboratories, investigators, and manufacturers of new tests in the process of FDA clearance.

RESULTS

Contracts and sample acquisition. Eleven contracts were awarded based on the response to a publically advertised funding announcement. The inclusion and exclusion criteria for selecting study participants varied based on the stage of Lyme disease or the look-alike disease for which the serum was sought. These criteria are outlined in Tables S1 to S3 in the supplemental material for each patient group and healthy controls. All patients were classified in two control categories: positive (Lyme disease patients) and negative (non-Lyme disease patients). Positive controls consisted of physician-diagnosed Lyme disease patients that were well documented, both clinically and epidemiologically. Negative controls were healthy residents of regions to which Lyme disease was endemic and regions to which it was not and persons with other diseases (infectious mononucleosis, fibromyalgia, multiple sclerosis, rheumatoid arthritis, syphilis, and severe periodontitis) that had no history of Lyme disease. The latter group of negative controls represents diseases that are reported to generate serologic cross-reactivity in antibody-based tests for Lyme disease (13, 14). These samples were targeted to enable assessment of Lyme disease serological tests' specificity. The control groups, subcategories, and numbers of serum samples collected for each are summarized in Table 1. A total of 471 serum samples were collected between 2008 and 2012. The volume of individual serum collected varied depending on the category and ranged from less than 10 ml to greater than 40 ml.

TABLE 1 Categories and numbers of serum samples collected

Category	No. of sera collected
Positive controls: Lyme disease	
Early Lyme disease with EM—acute phase	40
Early Lyme disease with EM—convalescent phase	38
Early disseminated Lyme carditis	7
Early disseminated Lyme neuroborreliosis	10
Late Lyme disease, Lyme arthritis	29
Negative controls: look-alikes	
Fibromyalgia	31
Infectious mononucleosis	30
Multiple sclerosis	22
Rheumatoid arthritis	21
Severe periodontitis	20
Syphilis	20
Negative controls: healthy	
Healthy, area of disease endemicity	101
Healthy, area of disease nonendemicity	102

Sample characterization based on medical record data. A medical questionnaire developed by contract providers and approved by the CDC was administered to all serum donors at the time they were enrolled in the study. Information collected through the questionnaire included age, sex, race, past medical history of tick-borne and Lyme disease, history of look-alike disease, and pregnancy, as well as any comorbidities and immunocompromising conditions. Exclusion criteria for enrollment of early Lyme patients included significant immunocompromising conditions, such as HIV or any disease that required immunosuppressive medications. Other immunocompromising conditions that were reported by the patient and did not fall into the category described above were recorded as part of their medical history.

Positive controls. (i) Early Lyme disease with EM. Selection of early Lyme disease patients for the LSR was based on the inclusion criteria described in Table S1 in the supplemental material. These criteria included appropriate epidemiological risk, the presence of a skin lesion consistent with EM of at least 5 cm in diameter, and when possible positive culture and/or PCR for *B. burgdorferi*. A

TABLE 2 PCR and culture results for patients with Lyme disease

Sample category and sample tested (n^a)	Culture: No. of patients tested	Culture: No. positive[b]	PCR: No. of patients tested	PCR: No. positive[b]
Early Lyme disease with EM—acute phase (40)	39	17	39	24
Blood and skin biopsy	30	15	27	17
Blood	9	2	10	5
Skin biopsy	0	0	2	2
Early disseminated Lyme carditis (7)	4	0	7	2
Blood and skin biopsy	1	0	1	0
Blood and heart biopsy	1	0	1	1
Blood	2	0	3	0
Skin biopsy	0	0	0	0
CSF[c] and blood	0	0	2	1
Early disseminated Lyme neuroborreliosis (10)	6	2	8	2
Blood and skin biopsy	1	1	0	0
Blood	5	1	7	1
Skin biopsy	0	0	1	1
Late disseminated Lyme disease with arthritis (29)			18	7
Joint fluid			18	7

[a] *n*, no. of patients.
[b] When two samples were tested from one patient, only one sample had to be positive to be counted as a positive result.
[c] CSF, cerebrospinal fluid.

total of 38 acute- and convalescent-phase serum pairs and 2 additional acute-phase samples were collected, for a total of 78 serum samples from 40 patients. Acute-phase samples were defined as baseline sera collected from patients during their first visit to the clinic and on the day of their Lyme disease diagnosis. The collection of sera from these patients occurred prior to newly prescribed antibiotic treatment. Convalescent-phase sera were collected between 10 to 35 days following the patients' initial diagnosis and after beginning treatment with antibiotics. The early Lyme disease patients presented with at least one EM rash (the number of EMs ranged from 1 to 27). The time between noticing an EM and collection of acute-phase sera ranged from 0 to 28 days, with an average of 7 days. Culture of blood and/or skin biopsy was performed by the provider and attempted with 39 of the 40 patients. In total, 17 of the 39 (44%) tested early Lyme disease patients were culture positive. Similarly, PCR was performed by the provider with samples from 39 of the 40 patients. Overall, 24 of the 39 (62%) patients had a positive PCR result. For the 40 enrolled patients, 26 (65%) were culture and/or PCR positive using one or more samples. Culture and PCR results are summarized in Table 2. At the time of their follow-up visit, 23 patients (58%) were classified as recovered. The remaining 17 (43%) patients had ongoing symptoms that consisted primarily of fatigue, joint pain, and headache.

(ii) Early disseminated Lyme disease with neuroborreliosis or Lyme carditis. Selection of neuroborreliosis (cranial nerve palsy, lymphocytic meningitis, or radiculopathy) and Lyme carditis (various degrees of heart block) patients for the LSR was based on appropriate epidemiological risk and objective clinical manifestations (see Table S1 in the supplemental material). Of the 17 patients enrolled, 10 presented with neuroborreliosis and 7 presented with Lyme carditis. Eight of the patients (47%) also had an EM or multiple EMs (up to 25) at the time of enrollment. The number of days between a patient's initial and follow-up visits ranged from 2 to 28 days.

Culture was performed by the provider with samples from 10 of 17 patients (4 Lyme carditis patients and 6 Lyme neuroborreliosis patients). Of these, only 2 (20%) of the 10 tested patients with Lyme neuroborreliosis were culture positive from one or more samples. PCR was also performed by the provider on samples from 15 patients (7 Lyme carditis patients and 8 Lyme neuroborreliosis patients). PCR was positive for 4 (27%) of these 15 patients. Overall, 5 of the 17 patients (29%) with disseminated infection were culture and/or PCR positive (Table 2). Of the 17 patients, only 2 (12%) were considered to have recovered at the time of their follow-up visits. Other patients had ongoing symptoms, including dizziness, headache, tingling, and/or ongoing palsy.

(iii) Late disseminated Lyme disease with arthritis. The selection of 29 patients with a diagnosis of Lyme arthritis for the LSR was based on inclusion criteria (see Table S1 in the supplemental material). Additionally, the patients of this group were required to be two-tiered positive, following the guidelines of the CDC and the State and Territorial Public Health Laboratory Directors (4).

The length of time between a patient reporting joint symptoms and the date of sampling ranged from 1.5 to 547 days. The joint affected for all 29 patients was the knee. One patient also had symptoms in the elbow and wrist joints. The degree of joint swelling ranged from mild to severe. For 25 of the 29 patients, the antibiotic treatment occurred 1.5 to 152 days prior to sample collection. Four patients were not treated prior to sample collection. The case definition for late disease required laboratory confirmation of infection. Therefore, all serum samples were tested by IgG EIA and IgG WB by the provider. All samples were positive by IgG EIA and by IgG WB. VlsE testing (15) was also performed by the provider and found to be positive for all patients. Joint fluid was collected and tested by PCR from 18 patients, and 7 (39%) of those tested were found to be positive for *B. burgdorferi* DNA (Table 2). Due to the laboratory confirmation required for these samples, selection bias toward reactive samples cannot be discounted.

Negative controls. (i) Healthy residents of regions to which Lyme disease is endemic and regions to which it is not. A total of 101 sera were collected from healthy area-of-endemicity controls (all residing in New York). Sera from 102 healthy persons residing in Texas, an area to which Lyme disease is not endemic, were also collected. Inclusion and exclusion criteria for both groups are outlined in Table S2 in the supplemental material.

(ii) Fibromyalgia. A total of 31 sera were collected from patients diagnosed with fibromyalgia based on the 1990 (16) and 2010 (17) criteria for diagnosing fibromyalgia. None of the patients reported a diagnosis of Lyme disease, although 17 (55%) patients had been previously tested for Lyme disease. Inclusion and exclusion criteria are outlined in Table S3 in the supplemental material.

(iii) Infectious mononucleosis. A total of 30 sera were collected from patients in Colorado that were diagnosed with infectious mononucleosis. All 30 patients had a positive rapid mononucleosis test using the Wampole MonoTest (Alere Inc.,

Waltham, MA) or a Mono-Latex test (Inverness Medical, Scarborough, ME). Only one (3%) patient reported a comorbidity (diabetes), and three (10%) patients were prescribed antibiotics for *Streptococcus* infection. Inclusion and exclusion criteria are outlined in Table S3 in the supplemental material.

(iv) Multiple sclerosis. Sera were collected from 22 patients that were diagnosed with multiple sclerosis. A diagnosis of multiple sclerosis followed the 2005 revisions of the "McDonald Criteria" (18) (see Table S3 in the supplemental material). Of these patients, 18 had relapsing-remitting multiple sclerosis and four had secondary-progressive multiple sclerosis. Patients reported anywhere from one to more than six multiple sclerosis episodes. Of these patients, none were in acute exacerbation at the time of sample collection. Patients were asked if they had current or past optic neuritis or myelitis. Five patients reported having optic neuritis, and 11 patients reported having myelitis (information was unknown for 2 patients).

Eighteen (82%) patients reported potential exposure to a tick bite. No patient reported a history of an EM rash, one patient reported a history of meningitis, one patient reported a history of facial nerve palsy, and two patients reported a history of radiculopathy. All information described above was unknown for one multiple sclerosis patient. Serologic testing, if known, was documented by the provider for antinuclear antibody (ANA), rheumatoid factor, and neuromyelitis optica antibody. Titers of ANA were found to be weakly positive for two patients and unknown for six patients. Rheumatoid factor was found to be weakly positive for one patient and unknown for seven patients. Neuromyelitis optica antibody was negative for 8 patients and unknown for 14 patients.

(v) Rheumatoid arthritis. A total of 21 sera were collected from patients that met the American College of Rheumatology (ACR) criteria for rheumatoid arthritis diagnosis (19) (see Table S3 in the supplemental material). Rheumatoid factor (RF) was positive for 17 patients, negative for 3 patients, and unknown for 1 patient. The presence of anti-cyclic citrullinated peptide (CCP) antibodies (19) was positive for 11 patients, negative for 3 patients, and unknown for 7 patients. Radiographs to identify erosions or bony decalcification localized in or adjacent to the involved joints were positive for 10 patients and negative for 11 patients. Additionally, ANA tests were positive for four patients.

(vi) Severe periodontitis. Twenty serum samples were collected from patients diagnosed with severe periodontitis. Diagnosis of severe periodontitis was based on the criteria set forth by the American Academy of Periodontology (20). These criteria are outlined in Table S3 in the supplemental material.

(vii) Syphilis. Twenty serum samples were collected from patients with active syphilis. All patients were diagnosed following the criteria outlined in Table S3 in the supplemental material. Of the patient samples collected, three (15%) were diagnosed with primary syphilis, four (20%) with secondary syphilis, 10 (50%) with early latent syphilis, and three (15%) with late latent syphilis.

Standard two-tiered testing. Samples received at CDC were given a unique identifier and assayed in a blinded manner using two-tiered serologic testing. The results of this testing are summarized in Table 3. Sera collected at baseline from acute-phase patients with early Lyme disease ($n = 40$) were found to be 68% positive by EIA, 35% positive by IgM WB, and 20% positive by IgG WB. A relatively small proportion (40%) of acute-phase patient samples was positive by standard two-tiered testing (positive or equivocal EIA followed by positive IgM and/or IgG WB). Convalescent-phase sera collected from these patients ($n = 38$) were 89% positive by EIA, 53% positive by IgM WB, 37% positive by IgG WB, and 68% positive by standard two-tiered testing without regard to the duration of illness prior to sample collection. CDC recommendations state that sera from patients collected more than 30 days after the onset of illness should not be tested by IgM WB to avoid false-positive results (4). Twelve of the 38 convalescent-phase samples were taken between 31 and 45 days following the onset of illness. Thus, based on the recommended IgM WB restriction, 61% of convalescent patient samples were positive by two-tiered testing.

Patients with early disseminated Lyme disease presenting with neuroborreliosis ($n = 10$) or Lyme carditis ($n = 7$) were 94% positive by EIA (9 neuroborreliosis and 7 carditis samples), 82% positive by IgM WB (10 neuroborreliosis and 4 carditis samples), 41% by IgG WB (3 neuroborreliosis and 4 carditis samples), and 88% positive (9 neuroborreliosis and 6 carditis samples) by two-tiered testing (IgM and/or IgG WB) with no 30-day IgM WB criteria applied. With the CDC-recommended IgM testing criteria used, 5 of these 17 patients fell beyond the $\leq$30-day criteria for use of IgM WB. However, these exclusions did not alter the two-tiered testing results for this patient group.

Sera from patients with late disseminated Lyme disease with arthritis ($n = 29$) were 100% positive by EIA, 31% positive by IgM WB, 100% positive by IgG WB, and 100% positive by two-tiered testing (IgM and/or IgG WB). Twenty patients from this group had a duration of illness beyond the $\leq$30-day criteria for use of IgM WB. Because all 29 patients were two-tiered positive by IgG WB, the IgM results with or without the 30-day criteria did not change the overall two-tiered results. It should be noted that positive two-tiered serology was a criteria for selection of patients with Lyme arthritis (see Table S1 in the supplemental material); therefore, it was expected that all samples be positive by two-tiered testing.

The Lyme disease-specific serologic reactivity of samples from negative controls ranged from nonreactive to highly reactive (Table 3). Sera from patients with severe periodontitis ($n = 20$) were nonreactive for Lyme disease by EIA and WB. Similarly, only 1 serum sample of those collected from patients with fibromyalgia ($n = 31$) was positive by IgM WB. Sera from patients with rheumatoid arthritis and multiple sclerosis had a higher cross-reactivity. Positive results were obtained by EIA with 10% and 18% of sera from patients with rheumatoid arthritis and multiple sclerosis, respectively. Low reactivity (5% positive) was observed for both of these groups in two-tiered testing using IgM WB and no 30-day cutoff. Application of the 30-day illness restriction for the use of IgM WB resulted in no false-positive two-tiered interpretations. The two groups with the highest cross-reactivity were syphilis and infectious mononucleosis. Sera from patients diagnosed with syphilis were 85% and 10% positive by the Lyme disease EIA and two-tiered testing using IgM WB, respectively. No syphilis patient samples were positive in IgG WB. However, for one syphilis patient with a disease duration of $<$30 days, the two-tiered test interpretation was positive. Sera from patients with infectious mononucleosis were 53% positive by EIA and 10% positive by two-tiered testing with or without IgM WB restrictions. Since most infectious mononucleosis patients who seek medical care will do so within 30 days of illness onset, IgM WBs will often be utilized if Lyme disease testing is performed, and this could lead to

TABLE 3 Two-tiered testing of sera

Sample category (n^a)	No. (%) positive by method					
		WB[c]		Two-tiered testing		
	WCS EIA (Vidas)[b]	IgM	IgG	Vidas-WB IgM[d]	Vidas-WB IgG	Standard[e]
Lyme disease, total patients = 86						
Early Lyme disease with EM						
Acute phase (40)	27 (68)	14 (35)	8 (20)	12 (30)	8 (20)	16 (40)
Convalescent phase (38)	34 (89)	20 (53)	14 (37)	20 (53)	13 (34)	23 (61)
Early Lyme disease with Lyme neuroborreliosis or Lyme carditis						
Lyme neuroborreliosis (10)	9 (90)	10 (100)	3 (30)	9 (90.0)	3 (30)	9 (90)
Lyme carditis (7)	7 (100)	4 (57)	4 (57)	4 (57)	4 (57)	6 (86)
Late Lyme diseases						
Lyme arthritis (29)	29 (100)	9 (31)	29 (100)	9 (31)	29 (100)	29 (100)
Look-alike diseases, total patients = 144						
Fibromyalgia (31)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)
Severe periodontitis (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Rheumatoid arthritis (21)	2 (10)	1 (5)	0 (0)	1 (5)	0 (0)	0 (0)
Syphilis (20)	17 (85)	2 (10)	0 (0)	2 (10)	0 (0)	1 (5)
Multiple sclerosis (22)	4 (18)	2 (9)	0 (0)	1 (5)	0 (0)	0 (0)
Infectious mononucleosis (30)	16 (53)	5 (17)	0 (0)	3 (10)	0 (0)	3 (10)
Healthy controls, total donors = 203						
Area of disease endemicity (101)	9 (9)	2 (2)	2 (2)	1 (1)	2 (2)	2 (2)
Area of disease nonendemicity (102)	5 (5)	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)

[a] *n*, no. of samples.
[b] WCS, whole-cell sonicate; EIA, enzyme immunoassay; Vidas, a Lyme IgM and IgG polyvalent assay (bioMérieux, Inc., Durham, NC).
[c] WB, Western blotting; IgM and IgG immunoblotting assays (MarDx Diagnostics, Inc., Carlsbad, CA).
[d] Positive IgM WB results are recorded regardless of the duration of illness prior to specimen collection.
[e] IgM WB results are recorded only when the duration of illness is <30 days.

misdiagnosis. For other patient groups (rheumatoid arthritis, fibromyalgia, multiple sclerosis, and severe periodontitis), all patients sought medical care >30 days after their initial symptoms began.

Sera from healthy persons residing in areas to which Lyme disease is endemic (n = 101) were 9% positive by EIA, and were 2% positive by two-tiered testing using the IgM WB or IgG WB. Sera from healthy persons residing in areas to which Lyme disease is not endemic (n = 102) were 5% positive by EIA, and all were negative by two-tiered testing (IgM and IgG WBs).

Composition of panels. Based on anticipated use of the sera and our previous experience with distribution of serum panels, we identified four general panel categories (Table 4). The first panel category is validation. This panel is composed of five positive Lyme patient samples and five healthy controls and is intended to validate commercial assays and instruments. The second panel category is research. Two panels fall under this category and are identified as Research I and Research II. The Research I panel consists of 32 sera, and Research II consists of 92 sera. For both of these panels, all three stages of Lyme disease, all of the Lyme disease look-alike diseases, and the healthy controls (in areas of disease endemicity and nonendemicity) are represented. The third panel category is premarketing. This panel is composed of 280 sera representing all three stages of Lyme disease, all of the Lyme disease look-alike diseases, and the healthy controls (in areas of disease endemicity and nonendemicity). The fourth panel category is custom. This panel will be assembled in response to more focused needs of individual investigators.

DISCUSSION

Assessment of the antibody response to *B. burgdorferi* infection has been the mainstay of laboratory confirmation of Lyme disease for more than 20 years. In the United States, a two-tiered serology algorithm was recommended in 1995 (4), and a similar strategy was established in Europe (21, 22). Although published studies frequently report serology test data on individual investigations with small patient numbers, fewer report on two-tiered test data obtained with large and well-characterized Lyme borreliosis patient sample sets. Among the later studies with U.S. patients, significant ranges in sensitivity are reported. Specifically, 29 to 40% sensitivity for the earliest acute-phase samples from EM patients, 29 to 78% sensitivity for convalescent-phase samples from these same patients, and 40 to 87% sensitivity for patients with early disseminated disease (Lyme neuroborreliosis and Lyme carditis) have been observed (8, 15, 23). In late-stage Lyme arthritis or Lyme neuroborreliosis, two-tiered sensitivity has been much higher and more tightly grouped, ranging from 97 to 100% (15, 23–25). Two-tiered variability in sensitivity with early Lyme disease is explained by inappropriate test use and differences in duration and dissemination of disease in tested patients (3, 26). Variability in sensitivity has also led to confusion and misunderstanding of the value of testing and how to interpret results.

Medical practitioners and the public at risk for Lyme disease, as well as test developers, would benefit from access to large sample sets and the corresponding sample data. Additionally, greater benefit would be gleaned from comparative data analyses using a stan-

TABLE 4 Composition of serum panels

Panel type (n^a)	Primary purpose	Sample	No. of samples for each disease/control type[b]
Validation (10)	Controls for validation of commercial assays and associated equipment	Early Lyme disease with EM—acute phase	2
		Lyme carditis or neuroborreliosis	1
		Lyme arthritis	2
		Controls, area of disease endemicity	3
		Controls, area of disease nonendemicity	2
Research I (32)	Research; proof-of-concept experiments of novel assays	Early Lyme disease with EM—acute phase	4
		Early Lyme disease with EM—convalescent phase	4
		Lyme carditis or neuroborreliosis	2
		Lyme arthritis	2
		Fibromyalgia	2
		Rheumatoid arthritis	2
		Multiple sclerosis	2
		Mononucleosis	2
		Syphilis	2
		Periodontitis	2
		Controls, area of disease endemicity	4
		Controls, area of disease nonendemicity	4
Research II (92)	Research; further development/improvement of novel assays	Early Lyme disease with EM—acute phase	10
		Early Lyme disease with EM—convalescent phase	10
		Lyme carditis or neuroborreliosis	6
		Lyme arthritis	6
		Fibromyalgia	6
		Rheumatoid arthritis	6
		Multiple sclerosis	6
		Mononucleosis	6
		Syphilis	6
		Periodontitis	6
		Controls, area of disease endemicity	12
		Controls, area of disease nonendemicity	12
Premarketing (280)	Premarketing test evaluation requiring large sample sets	Early Lyme disease with EM—acute phase	30
		Early Lyme disease with EM—convalescent phase	30
		Lyme carditis or neuroborreliosis	10
		Lyme arthritis	20
		Fibromyalgia	15
		Rheumatoid arthritis	15
		Multiple sclerosis	15
		Mononucleosis	15
		Syphilis	15
		Periodontitis	15
		Controls, area of disease endemicity	50
		Controls, area of disease nonendemicity	50
Custom (as needed)	As determined by user	As needed	As needed

[a] *n*, total no. of samples.
[b] The total number of serum samples listed is only an example and may change as needed.

dardized sample set that includes healthy and disease controls that are likely to be encountered in normal test use. Such panels are time and cost expensive and require carefully formulated inclusion and exclusion criteria to maximize their utility. The CDC LSR was developed to address these needs and limitations. Specifically, this repository consists of sera from positive controls that include all stages of Lyme disease meeting the CDC surveillance criteria and negative controls that include healthy donors from disease-endemic and non-disease-endemic areas and patients with look-alike diseases. Use of these samples will enable comparative assessment among novel diagnostic tests and with those already in use.

In addition to improving the sensitivity of diagnostics for Lyme disease, the specificity of new tests must also be considered. The specificity of two-tiered testing for Lyme disease beyond 30 days of duration has generally been reported to be 98 to 100% (1, 8, 15, 23). However, this specificity is dependent on the negative-control sera used. The panels described here offer sera from patients with look-alike diseases (fibromyalgia, infectious mononucleosis, multiple sclerosis, rheumatoid arthritis, syphilis, and severe periodontitis) that are documented as causing cross-reactivity in serologic assays for Lyme disease (27, 28) and/or are a differential diagnosis for at least one of the stages of Lyme disease (2). Syphilis and some cases of severe periodontitis are caused by the spirochetes *Treponema pallidum* and *Treponema denticola*, respectively, and anti-

bodies to both organisms have been shown to be cross-reactive with *Borrelia* antigens (29). No cross-reactivity to the Lyme disease EIA and two-tiered test was observed with sera from patients with severe periodontitis. Sera from patients with syphilis, however, had relatively high cross-reactivity (85% of samples) when tested by whole-cell EIA (first tier). When two-tiered testing was performed, the specificity with these patient samples increased to 95%. Infectious mononucleosis was included in the LSR panel because it is an acute infection that generates a clinical picture that may be similar to early Lyme disease and an antibody response that can be cross-reactive in diagnostic assays for Lyme disease (30). In this study, 53% of sera from patients with infectious mononucleosis and no history or recent risk of Lyme disease showed cross-reactivity with borrelial antigens by the EIA, and 10% showed cross-reactivity by two-tiered testing. Additionally, 18% of sera from patients with multiple sclerosis, a disease that causes nervous system complications similar to those in Lyme neuroborreliosis (2), were false positive when tested by first-tier EIA. However, specificity improved to 100% when two-tiered testing was applied to these patient samples. To account for differential diagnosis for Lyme arthritis, sera from patients with fibromyalgia and rheumatoid arthritis were collected and tested. Limited cross-reactivity with EIA or two-tiered testing was observed for patients in these last groups.

Sera from healthy donors in regions to which Lyme disease is endemic and not endemic were collected and included in the LSR. Two-tiered testing of these sera found that healthy controls living in areas of endemicity and nonendemicity for Lyme disease yielded specificities of 98% and 100%, respectively. This is in line with previous reports for these two sample groups (15, 23).

The first- and second-tier Lyme disease testing results reported here support the combined sensitivity and specificity rationale of the two-tiered algorithm as opposed to use of just one test. However, alternative and FDA-cleared first- and second-tier tests with various performance characteristics have been described (23, 25, 31). The LSR has been evaluated with additional assays (C6 EIA and VlsE), and those findings are available to users of the repository.

While a number of negative-control categories with overlapping clinical and/or antibody profiles were sought and included in the LSR, other relevant negative-control disease categories, including summer flu, other tick-borne diseases, and hepatitis, were not obtained. Similarly, samples were not obtained from patients who acquired Lyme disease outside the United States. Thus, infections with *Borrelia garinii* and *Borrelia afzelii* were not represented. Use of the LSR panels, therefore, should be considered partial fulfillment of sample inclusion in the process of new Lyme disease test development, validation, and FDA clearance.

LSR users will have access to a free resource of sera from well-characterized patients. Panels will be supplied to investigators in a progressive manner, with Research Panel I being the first provided and access to additional panels (Research II or custom panels) based on use and data generated from earlier panels. In addition to the availability of sera in the form of research panels, a validation panel will be available to laboratories needing a few positive and negative controls for instrument validation. To obtain a panel, requests should be made to the Division of Vector-Borne Diseases, Bacterial Diseases Branch.

ACKNOWLEDGMENTS

We thank the National Institutes of Health for their support for this project under interagency title CDC-NIH Lyme Disease Clinical Sample Repository and funding number Y1-A1-8362-01.

We thank the sample providers and support staff for their contributions.

REFERENCES

1. **Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP.** 2005. Diagnosis of Lyme borreliosis. Clin. Microbiol. Rev. **18:**484–509. http://dx.doi.org/10.1128/CMR.18.3.484-509.2005.
2. **Stanek G, Wormser GP, Gray J, Strle F.** 2012. Lyme borreliosis. Lancet **379:**461–473. http://dx.doi.org/10.1016/S0140-6736(11)60103-7.
3. **Aguero-Rosenfeld ME.** 2008. Lyme disease: laboratory issues. Infect. Dis. Clin. North Am. **22:**301–313. http://dx.doi.org/10.1016/j.idc.2007.12.005.
4. **Centers for Disease Control and Prevention.** 1995. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Morb. Mortal. Wkly. Rep. **44:**590–591.
5. **Feder HM, Johnson BJ, O'Connell S, Shapiro ED, Steere AC, Wormser GP, Agger WA, Artsob H, Auwaerter P, Dumler JS, Bakken JS, Bockenstedt LK, Green J, Dattwyler RJ, Munoz J, Nadelman RB, Schwartz I, Draper T, Mcsweegan E, Halperin JJ, Klempner MS, Krause PJ, Mead P, Morshed M, Porwancher R, Radolf JD, Smith RP, Jr, Sood S, Weinstein A, Wong SJ, Zemel L.** 2007. A critical appraisal of "chronic Lyme disease." N. Engl. J. Med. **357:**1422–1430. http://dx.doi.org/10.1056/NEJMra072023.
6. **Weinstein A.** 2008. Laboratory testing for Lyme disease: time for a change? Clin. Infect. Dis. **47:**196–197. http://dx.doi.org/10.1086/589316.
7. **Sivak SL, Aguero-Rosenfeld ME, Nowakowski J, Nadelman RB, Wormser GP.** 1996. Accuracy of IgM immunoblotting to confirm the clinical diagnosis of early Lyme disease. Arch. Intern. Med. **156:**2105–2109. http://dx.doi.org/10.1001/archinte.1996.00440170121013.
8. **Nowakowski J, Schwartz I, Liveris D, Wang G, Aguero-Rosenfeld ME, Girao G, McKenna D, Nadelman RB, Cavaliere LF, Wormser GP, Lyme Disease Study Group.** 2001. Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. Clin. Infect. Dis. **33:**2023–2027. http://dx.doi.org/10.1086/324490.
9. **Liveris D, Schwartz I, Bittker S, Cooper D, Iyer R, Cox ME, Wormser GP.** 2011. Improving the yield of blood cultures from patients with early Lyme disease. J. Clin. Microbiol. **49:**2166–2168. http://dx.doi.org/10.1128/JCM.00350-11.
10. **Liveris D, Schwartz I, McKenna D, Nowakowski J, Nadelman RB, DeMarco J, Iyer R, Cox ME, Holmgren D, Wormser GP.** 2012. Quantitation of cell-associated borrelial DNA in the blood of Lyme disease patients with erythema migrans. Eur. J. Clin. Microbiol. Infect. Dis. **31:**791–795. http://dx.doi.org/10.1007/s10096-011-1376-x.
11. **Liveris D, Schwartz I, McKenna D, Nowakowski J, Nadelman R, Demarco J, Iyer R, Bittker S, Cooper D, Holmgren D, Wormser GP.** 2012. Comparison of five diagnostic modalities for direct detection of *Borrelia burgdorferi* in patients with early Lyme disease. Diagn. Microbiol. Infect. Dis. **73:**243–245. http://dx.doi.org/10.1016/j.diagmicrobio.2012.03.026.
12. **Li X, McHugh G, Damle ND, Sikand VK, Glickstein L, Steere AC.** 2011. Burden and viability of *Borrelia burgdorferi* in skin and joints of patients with erythema migrans. Arthritis Rheum. **63:**2238–2247. http://dx.doi.org/10.1002/art.30384.
13. **Fawcett PT, Gibney KM, Rose CD, Dubbs SB, Doughty RA.** 1992. Frequency and specificity of antibodies that crossreact with *Borrelia burgdorferi* antigens. J. Rheum. **19:**582–597.
14. **Bruckbauer HR, Preac-Mursic V, Fuchs R, Wilske B.** 1992. Cross-reactive proteins of *Borrelia burgdorferi*. Eur. J. Clin. Microbiol. Infect. Dis. **11:**224–232. http://dx.doi.org/10.1007/BF02098084.
15. **Branda JA, Aguero-Rosenfeld ME, Ferraro MJ, Johnson BJ, Wormser GP, Steere AC.** 2010. 2-tiered antibody testing for early and late Lyme disease using only an immunoglobulin G blot with the addition of a VlsE band as the second-tier test. Clin. Infect. Dis. **50:**20–26. http://dx.doi.org/10.1086/648674.
16. **Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, Tugwell P, Campbell SM, Abeles M, Clark P.** 1990. The American College of Rheumatology1990 criteria for the classification of

fibromyalgia: report of the Multicenter Criteria Committee. Arthritis Rheum. **33:**160–172. http://dx.doi.org/10.1002/art.1780330203.
17. **Wolfe F, Clauw DJ, Fitzcharles MA, Goldenberg DL, Katz RS, Mease P, Russell AS, Russell IJ, Winfield JB, Yunus MB.** 2010. The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. Arthritis Care Res. (Hoboken) **62:**600–610. http://dx.doi.org/10.1002/acr.20140.
18. **Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, Lublin FD, Metz LM, McFarland HF, O'Connor PW, Sandberg-Wollheim M, Thompson AJ, Weinshenker BG, Wolinsky JS.** 2005. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria." Ann. Neurol. **58:**840–846. http://dx.doi.org/10.1002/ana.20703.
19. **Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, III, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JMW, Hobbs K, Huizinga TWJ, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Menard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovský J, Wolfe F, Hawken G.** 2010. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann. Rheum. Dis. **69:**1580–1588. http://dx.doi.org/10.1136/ard.2010.138461.
20. **American Academy of Periodontology.** 2000. Parameter on aggressive periodontitis. J. Periodontol. **71:**867–869. http://dx.doi.org/10.1902/jop.2000.71.5-S.867.
21. **Brouqui P, Bacellar F, Baranton G, Birtles RJ, Bjoërsdorff A, Blanco JR, Caruso G, Cinco M, Fournier PE, Francavilla E, Jensenius M, Kazar J, Laferl H, Lakos A, Lotric Furlan S, Maurin M, Oteo JA, Parola P, Perez-Eid C, Peter O, Postic D, Raoult D, Tellez A, Tselentis Y, Wilske B, ESCMID Study Group on Coxiella Anaplasma Rickettsia and Bartonella, European Network for Surveillance of Tick-Borne Diseases.** 2004. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. Clin. Microbiol. Infect. **10:**1108–1132. http://dx.doi.org/10.1111/j.1469-0691.2004.01019.x.
22. **Wilske B, Fingerle V, Schulte-Spechtel U.** 2007. Microbiological and serological diagnosis of Lyme borreliosis. FEMS Immunol. Med. Microbiol. **49:**13–21. http://dx.doi.org/10.1111/j.1574-695X.2006.00139.x.
23. **Bacon RM, Biggerstaff BJ, Schriefer ME, Gilmore RD, Philipp MT, Steere AC, Wormser GP, Marques AR, Johnson BJ.** 2003. Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. J. Infect. Dis. **187:**1187–1199. http://dx.doi.org/10.1086/374395.
24. **Steere AC, McHugh G, Damle N, Sikand VK.** 2008. Prospective study of serologic tests for Lyme disease. Clin. Infect. Dis. **47:**188–195. http://dx.doi.org/10.1086/589242.
25. **Branda JA, Linskey K, Kim YA, Steere AC, Ferraro MJ.** 2011. Two-tiered testing for Lyme disease with the use of 2 enzyme immunoassays, a whole-cell sonicate enzyme immunoassay followed by a VlsE C6 peptide enzyme immunoassay. Clin. Infect. Dis. **53:**541–547. http://dx.doi.org/10.1093/cid/cir464.
26. **Aguero-Rosenfeld ME, Nowakowski J, Bittker S, Cooper D, Nadelman RB, Wormser GP.** 1996. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J. Clin. Microbiol. **34:**1–9.
27. **Magnarelli LA, Miller JN, Anderson JF, Riviere GR.** 1990. Cross-reactivity of nonspecific treponemal antibody in serologic tests for Lyme disease. J. Clin. Microbiol. **28:**1276–1279.
28. **Burkot TR, Schriefer ME, Larsen SA.** 1997. Cross-reactivity to *Borrelia burgdorferi* proteins in serum samples from residents of a tropical country nonendemic for Lyme disease. J. Infect. Dis. **175:**466–469. http://dx.doi.org/10.1093/infdis/175.2.466.
29. **Luft BJ, Dunn JJ, Dattwyler RJ, Gorgone G, Gorevic PD, Schubach WH.** 1993. Cross-reactive antigenic domains of the flagellin protein of *Borrelia burgdorferi*. Res. Microbiol. **144:**251–257. http://dx.doi.org/10.1016/0923-2508(93)90009-Q.
30. **Magnarelli LA.** 1995. Current status of laboratory diagnosis for Lyme disease. Am. J. Med. **98:**10S–14S. http://dx.doi.org/10.1016/S0002-9343(99)80039-4.
31. **Johnson JBJ.** 2011. Laboratory diagnostic testing for *Borrelia burgdorferi* infection, p 73–88. *In* Halperin JJ (ed), Lyme disease: an evidence-based approach. CAB International, Oxfordshire, United Kingdom.