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Article

Evaluation of Fifteen Commercially Available Serological Tests for Diagnosis of Lyme Borreliosis

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Abstract The performance of 11 commercially available enzyme immunoassays (EIA) and four Western blot (WB) tests for the detection of IgM and IgG antibodies against *Borrelia burgdorferi* were compared. A total of 229 serum specimens were used: 26 from patients with early Lyme borreliosis, 13 from patients with late Lyme borreliosis, 62 from healthy controls and 128 from patients with disorders clinically mimicking Lyme borreliosis and/or known to cause cross-reactivity in Lyme borreliosis serological tests (patient control group). In specimens from patients with early Lyme borreliosis, the sensitivity of the individual tests ranged from 35 to 81% for detection of IgM. In late Lyme borreliosis, sensitivity of the tests ranged from 46 to 92%. In healthy controls the specificity of the tests ranged from 89 to 100% and from 82 to 97% for IgM and IgG tests, respectively. In the patient control group, specificity of the tests ranged from 75 to 90% for IgM and from 84 to 100% for IgG tests. The Behring (Germany) and Genzyme Virotech (Germany) IgM EIA tests showed the best performance in detecting early Lyme borreliosis. For the detection of late Lyme borreliosis, the Dako (Denmark) IgG test was the best despite its low sensitivity. The maximum sensitivity of Western blotting for detecting IgM in patients with early Lyme borreliosis and IgG in patients with late Lyme borreliosis was 50 and 46%, respectively. The use of an EIA-WB two-test protocol improved the specificity and positive predictive values of the EIA results but caused a significant loss in sensitivity. Patients with Epstein-Barr virus or cytomegalovirus infection who had a positive reaction in the IgM EIA could not be discriminated from patients with early Lyme borreliosis with the help of Western blotting. Hence, positive and negative predictive values in combination with sensitivity and specificity values indicated that the exclusion of these infections was more relevant than the confirmation of a positive IgM EIA with Western blot.

Introduction

Since the discovery in 1982 of *Borrelia burgdorferi* as the causative agent of Lyme borreliosis (LB) [1] and the subsequent awareness of the extended distribution of this infection [2], the development of serological tests to aid the diagnosis of LB has received much attention. Several factors have contributed to this development. Firstly, LB is a multisystemic disorder that may affect several organ systems such as the skin, nervous system, joints and/or heart. By virtue of its wide range of clinical manifestations, LB may mimick several other diseases [3, 4]. Hence, diagnosing the disease purely on clinical grounds may be difficult, especially in the absence of erythema migrans (EM) or a tick bite in the anamnesis. Moreover, EM only occurs in about 60% of patients [3, 4]. Secondly, the classical methods for confirming the diagnosis of a bacterial infection, i.e., isolation or direct visualization of the causative agent [5–7], and newer methods such as antigen detection, T-lymphocyte assay or polymerase chain reaction are either not sensitive enough or not readily available in clinical practice [8–23].

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Currently, enzyme immunosorbent assays (EIAs) are the most reliable and readily available laboratory tests for LB. However, there are several problems in the serodiagnosis of LB. False-negative results in serological tests can be attributed to the slow antibody response in LB; IgM antibodies may not be detectable until 6 weeks after disease onset, and production of IgG antibodies may follow within a few weeks but may not reach a peak until months after disease onset [4]. Antibiotic therapy given during early Lyme borreliosis (ELB) may prevent the development of antibodies [4]. Moreover, while the incidence of seronegative LB cases remains controversial, it has been estimated to be as high as 5% [24, 25]. False-positive results may occur due to cross-reactivity with antibodies against other microorganisms, or due to the presence of aspecific antibodies in various viral and autoimmune syndromes [4]. Furthermore, the occurrence of asymptomatic LB with positive test results in healthy controls is well documented [26, 27].

To improve sensitivity, especially in the early stages of the disease, and to reduce cross-reactivity, several methods have been advocated. These include the use of purified outer membrane, flagella or recombinant antigens and the preadsorption of sera with *Treponema phagedenis* or other bacterial antigens [28]. However, experience thus far has been variable. None of the currently available diagnostic methods including immunofluorescent antibody assays, EIA, hemagglutination assays and Western blot (WB), have been standardized with regard to performance, relevance, techniques, antigen preparations, reagents or even the definition of what constitutes a positive result [28, 29]. Despite these shortcomings, many laboratories follow the guidelines established by the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) and the U.S. Centers for Disease Control and Prevention (CDC), who recommend the use of a two-test protocol for the serodiagnosis of LB. The two-test protocol consists of screening with an IgM and/or IgG EIA followed by the use of IgM and/or IgG immunoblotting for reactive sera as a confirmatory test. Immunoblotting makes it feasible to visualize the immune response against individual *Borrelia* proteins.

Unfortunately, in many studies interpretation of immunoblot patterns has been subjective, which has resulted in diverse criteria for positivity [30–33]. Moreover, in most studies tests are evaluated based solely on sensitivity and specificity, which only characterize the validity of the test. Positive and negative predictive values are more relevant for clinical decision-making because they take into account the prevalence of the disease in a certain population.

In the present study, we evaluated 11 commercially available EIA tests, four Western blot (WB) tests and the EIA-WB two-test protocol using sera of confirmed LB patients as positive controls and sera of healthy persons as well as of patients with infections mimicking LB or known to cross-react in LB serological tests as negative controls.

Materials and Methods

Sera. Two hundered twenty-nine serum samples were collected in the Netherlands during a period of 1 year. Using clinical criteria, they were divided into three major groups according to their origin. The Lyme patient group consisted of 26 patients with early Lyme borreliosis who had presented with an erythema migrans and had a history of a tick bite and/or isolation of *Borrelia burgdorferi* from their skin lesions (ELB group) and 13 patients with late Lyme borreliosis (LLB group), four with acrodermatitis chronica atrophicans and nine with neuroborreliosis. All Lyme patients were included in the study irrespective of the results obtained in serological tests and all recovered after adequate antibiotic therapy. There was no occurrence of relapse in the group. The healthy control group consisted of 62 healthy controls with no history of LB and tick exposure. The patient control group consisted of 128 persons: 24 with Epstein-Barr virus (EBV) infection, 12 with acute cytomegalovirus (CMV) infection, 24 with rheumatoid factor-positive rheumatoid arthritis (RF), 24 with antinuclear antibodies (ANA), 23 with syphilis (TP) and 21 which were seropositive for the human immunodeficiency virus (HIV).

All Lyme patients had negative test results for RF (Eurogenetics, Belgium), ANA (immunofluorescent assay with Hep-2 cells, internal house test), EBV (Paul en Bunell, internal house test, and EBV-EIA, IgM, Genzyme Virotech, Germany), CMV (Abbott, USA), HIV (Abbott, USA) and specific syphilis antibodies (Fujirebio, Japan).

IgM and IgG Enzyme Immunoassays. The following 11 commercially available EIA tests were evaluated: Behring (Germany) IgM and IgG EIA, Boehringer (Germany) IgM and IgG EIA, Dako (Denmark) IgM and IgG EIA, Genzyme Virotech (Germany) IgM and IgG EIA, IBL (Germany) IgM and IgG EIA, and Milenia (Germany) IgM+IgG EIA. All kits were purchased at the same time and were lot consistent. All systems were used according to the instructions of the manufacturers. Test characteristics and types of antigens used are shown in Table 1. All samples were tested in duplicate. For the calculation of sensitivity and specificity, borderline results were considered positive. For further comparison of the EIAs, WBs and EIA-WB two-test protocol, indexes and predictive values for positive and negative results were calculated. The predictive values were derived from the likelihood-ratio using Baye's theorem [34] and listed for the IgM and IgG tests with a 20% prevalence of, respectively, ELB and LLB in the population combined with healthy controls (population I), non-Lyme patient controls (population II) and non-Lyme patient controls eliminated from the EBV and CMV control group (population III). The postulated 20% prevalence of LB in this study was based on the prevalence of infection with *Borrelia burgdorferi* in patients tested for LB in north and central Europe [35]. The prevalence varied from 15% (Poland) to 28% (Netherlands). Because of earlier findings that CMV and EBV infections cause false-positive results in IgM EIA-WB two-test protocols for ELB, the CMV and EBV control group was excluded in population III. For the calculation of intra-assay precision, the deviation of the duplicate values within 25% of the mean baseline was used as described in [36].

IgM and IgG Western Blots. Additionally, four commercially available WBs were tested: Genzyme Virotech (Germany) IgM and IgG and MRL (USA) IgM and IgG. All kits were purchased at the same time and were lot consistent. The 93/83 kDa, 41 kDa (Fla), 34 kDa (OspB), 31 kDa (OspA) and 23 kDa (OspC) bands

Table 1 Main characteristics of 11 enzyme immunoassay (EIA) kits and four Western blot (WB) kits for the detection of IgM and/or IgG antibodies for Lyme borreliosis

Test	Assay design	*B. burgdorferi* strain	Antigen preparation	Preabsorption of sera[c]	Total incubation time (min)[d]
Behring EIA	indirect EIA[a,b]	Pko	100 kDa, 41 kDa OspC, 17 kDa	yes	90
Boehringer EIA	indirect EIA[a,b]	Pko	100 kDa[b] 41 kDa (B31)[a] 41i kDa[a,b] Osp C (recomb)[a,b]	recommended	100
Dako EIA	μ capture[a]	DK	41 kDa	no	130
	indirect EIA[b]	DK	41 kDa	no	130
Genzyme Virotech EIA	indirect EIA[a,b]	2591	total sonicate	recommended	90
IBL, EIA	indirect EIA[a,b]	unknown	14 kDa (recomb)	yes	100
Milenia EIA	indirect EIA[a,b]	*B. afzelii*	total sonicate	no	70
Genzyme Virotech WB	WB[a,b]	2591	total sonicate	recommended	100
MRL WB	WB[a,b]	*B. garinii*	total sonicate	no	95

[a] IgM
[b] IgG
[c] Absorption with *Treponema phagedenis*
[d] Recomb, recombinant, serum, conjugate and substrate incubation

were identified with monoclonal antibodies (Dr Kramer, University Heidelberg, Department of Immunology, Heidelberg, Germany). For the identification of OspC in the MRL WB, a monospecific chicken IgY was used. With the exception of the band evaluation, the tests were performed according to the instructions of the manufacturers.

To ensure an objective band detection, a densitometrical approach was used. All blots were scanned with a 600 dpi flatbed scanner (E6; Microtek, Germany) and band intensities were integrated with the Gel-Pro Analyzer densitometrical software (Media Cybernetics, USA). The software generated Gaussian curves for each peak, and the contribution of one peak to a nearby peak was measured. After molecular weights were indicated, the software identified molecular weight, maximum optical density and area under the curve of each peak. Using the described protocol, band areas were determined for all bands in increments of 1 kDa. Intrablot variation was checked by comparing band areas of a seropositive patient [reactive to p93/83, p41 (Fla), p39 (BmpA), p34 (OspB), p31 (OspA) and p23 (OspC) antigens] performed at the start and end position of the blot kit. Blot kits with an intrablot variation higher than 15%, which occurred once in the MRL WB, were discarded and samples were retested. Interblot variation of band areas were corrected by applying a correction factor. The band area ratio of a reference band and corresponding band of strip 1 was used as a kit-specific correction factor. The reference band was defined as follows: a strip was incubated with the serum of a positive patient (the same as that used for the intrablot variation test), and a band with band area in the linear range of the densitometer was used as the reference. A kit-independent baseline value for positive band interpretation was applied. Baseline was set at a band area resulting in a maximum number of positive reactive ELB and LLB in IgM and IgG, respectively, and a minimum number of positive reactive healthy controls. For the calculations of sensitivity and specificity, borderline results were considered positive. The Genzyme Virotech IgM and IgG WB was interpreted as borderline when one and positive when minimum two of the following proteins were recognized: 83 kDa, 39 kDa, 34 kDa (OspB), 31 kDa (OspA), 25 kDa, 22 kDa (OspC), 20 kDa or 18 kDa.

The MRL IgM WB was positive when reactivity to the 23 kDa (OspC) and/or the 39 kDa was observed. The IgG MRL WB was positive when four or more of the 93 kDa, 45 kDa, 41 kDa, 39 kDa, 37 kDa, 23 kDa (OspC) and 21 kDa proteins showed reactivity.

For the calculation of the predictive values of the different tests, the sera were clustered in three populations. Population I consisted of the sera of LB patients and healthy control patients, population II contained the sera of LB patients and non-LB control patients, and population III consisted of the sera of LB patients and non-LB control patients with exclusion of EBV- and CMV-positive sera. The predictive values listed were derived from the likelihood-ratio using Baye's theorem [34] for an LB prevalance of 20%, as can be expected in clinical laboratory situations.

Results

Performance of Enzyme Immunoassays. Sensitivity and specificity data of the six EIA tests for the detection of IgM and IgG antibodies for LB are listed in Tables 2 and 3, respectively. Positive and negative predictive values for the detection of IgM antibodies in ELB and IgG antibodies in LLB, both calculated under an assumption of an LB prevalence of 20%, are presented in Table 4. Intra-assay variances of the six EIAs for the detection of IgM and IgG antibodies to Lyme borreliosis are listed in Table 5.

IgM Enzyme Immunassay. As shown in Table 2, sensitivity for the detection of IgM antibodies by EIA in ELB ranged from 81 to 31%. Sensitivity for detection of LLB varied from 69 to 46%. Specificity ranged from 100 to 90% in the 62 healthy controls and from 89 to 75% in the patient control group. A drop in specificity in the non-Lyme patient controls was caused mainly by the false-positive results of sera from EBV and CMV patients, except for the Dako test, where a decrease in specificity occurred only in the EBV control group. Other non-Lyme groups in which the specificity was lower than 90% were HIV patients in the Genzyme Virotech test and syphilis patients in the IBL and Milenia tests. Excluding EBV- and CMV-positive sera from the non-Lyme patient control group resulted in a

Table 2 Sensitivity and specificity (%) of six enzyme immunoassays (EIAs) and two Western blots (WBs) for the detection of IgM antibodies for Lyme borreliosis

Test	Percent sensitivity		Percent specificity								
	ELB ($n=26$	LLB ($n=13$)	Healthy controls ($n=62$)	Non-Lyme patients controls							
				(1–6) Non-Lyme patients ($n=128$)	(3–6) Non-Lyme patients without EBV/CMV ($n=92$)	(1) EBV ($n=24$)	(2) CMV ($n=12$)	(3) RF ($n=24$)	(4) ANA ($n=24$)	(5) TP ($n=23$)	(6) HIV ($n=21$)
Behring EIA	77	62	98	85	98	38	83	96	100	100	95
Boehringer EIA	35	46	100	89	99	58	75	100	100	100	95
Dako EIA	65	69	95	88	99	46	92	100	100	100	95
Genzyme Virotech EIA	81	62	98	81	92	42	67	96	96	91	86
IBL EIA	65	62	90	75	89	33	50	92	96	78	90
Milenia EIA[a]	31	69	95	88	88	92	83	96	96	70	90
Genzyme Virotech WB	50	62	89	80	82	58	58	88	96	78	95
MRL WB	46	54	98	90	98	83	42	92	100	100	100

[a] IgM + IgG; ELB, early Lyme borreliosis; LLB late lyme borreliosis; EBV, Epstein-Barr virus; CMV, cytomegalovirus; RF, rheumatoid factor-positive rheumatoid arthritis; ANA, antinuclear antibodies; TP, syphilis

Table 3 Sensitivity and specificity (%) of six enzyme immunoassays (EIAs) and two Western blots (WBs) for the detection of IgG antibodies for Lyme borreliosis

Test	Percent sensitivity		Percent specificity								
	ELB ($n=26$	LLB ($n=13$)	Healthy controls ($n=62$)	Non-Lyme patients controls							
				(1–6) Non-Lyme patients ($n=128$)	(3–6) Non-Lyme patients without EBV/CMV ($n=92$)	(1) EBV ($n=24$)	(2) CMV ($n=12$)	(3) RF ($n=24$)	(4) ANA ($n=24$)	(5) TP ($n=23$)	(6) HIV ($n=21$)
Behring EIA	69	92	85	84	86	79	75	83	96	78	86
Boehringer EIA	38	54	89	89	97	88	100	92	96	78	86
Dako EIA	50	77	97	98	98	100	92	96	100	100	95
Genzyme Virotech EIA	54	92	94	92	89	96	100	100	92	70	95
IBL EIA	46	69	87	86	78	88	92	92	92	87	67
Milenia EIA[a]	31	69	95	88	88	92	83	96	96	70	90
Genzyme Virotech WB	27	46	82	91	89	100	83	88	96	96	76
MRL WB	4	46	97	100	100	100	100	100	100	100	100

[a] IgM + IgG; ELB, early Lyme borreliosis; LLB late lyme borreliosis; EBV, Epstein-Barr virus; CMV, cytomegalovirus; RF, rheumatoid factor-positive rheumatoid arthritis; ANA, antinuclear antibodies; TP, syphilis

specificity higher than 90% for all tests except the IBL and Milenia tests.

As shown in Tables 2 and 4, the two tests with the highest sensitivity for detection of ELB, i.e. Genzyme Virotech (81%) and Behring (77%), had a positive and negative predictive value higher than 90% in population I (healthy controls and an ELB prevalence of 20%). In population II (control patients with disorders other than LB with an ELB prevalence of 20%), Behring and Genzyme Virotech tests showed a positive predictive value of 56% and 52%, respectively. Excluding CMV and EBV patients from the non-Lyme patient controls (group III, Table 4) increased the positive predictive value to 91% and 72% for the Behring and Genzyme Virotech tests, respectively. In all control groups, mean index values for positive results were more than twice the baseline, except for the Milenia IgM EIA, which scored less than twice the baseline in the EBV, ANA, RF and TP control groups. The Dako IgM EIA scored more than four times the baseline in the scored groups and more than 12 times the baseline for the LLB group.

As shown in Table 5, the intra-assay precision was higher than 90% for all IgM EIAs tested.

Table 4 Positive and negative predictive values of six enzyme immunoassays (EIAs) and two Western blots (WBs) for the detection of IgM or IgG antibodies in early Lyme borreliosis (ELB) and late Lyme borreliosis (LLB) in three different populations. Predictive value is calculated for a prevalence of 20% early Lyme borreliosis and 20% late Lyme borreliosis in the population tested in IgM and IgG, respectively

		Population I[a]		Population II[b]		Population III[c]	
		PPV %	NPV %	PPV %	NPV %	PPV %	NPV %
Behring EIA	IgM	92	94	56	94	91	94
	IgG	61	98	59	98	62	98
Boehringer EIA	IgM	100	86	44	84	90	86
	IgG	54	88	55	89	82	89
Dako EIA	IgM	77	92	58	91	94	92
	IgG	86	94	91	94	91	94
Genzyme Virotech EIA	IgM	93	95	52	94	72	95
	IgG	78	98	74	98	68	98
IBL EIA	IgM	63	91	40	90	60	91
	IgG	57	92	55	92	44	91
Milenia EIA[d]	ELB	61	85	39	84	39	84
	LLB	78	92	59	92	59	92
Genzyme Virotech WB	IgM	53	88	38	86	41	87
	IgG	39	86	56	87	51	87
MRL WB	IgM	85	88	53	87	85	88
	IgG	79	88	100	88	100	88

[a] Lyme borreliosis and healthy control group
[b] Lyme borreliosis and non-Lyme patient control group
[c] Lyme borreliosis and non-Lyme patient control group without cytomegalovirus and Epstein-Barr virus patient controls
[d] IgM + IgG
PPV, positive predictive value; NPV, negative predictive value

Table 5 Comparison of intra-assay precision of six enzyme immunoassays (EIAs) for the detection of antibodies to *Borrelia burgdorferi*

EIA	No. (%) of IgG tested		No. (%) of IgM tested	
Behring	17	(96)	41	(98)
Boehringer	24	(95)	45	(96)
Dako	9	(93)	14	(93)
Genzyme Virotech	20	(91)	33	(98)
IBL	41	(97)	36	(97)
Milenia[a]	49	(96)	49	(96)

[a] IgM + IgG

IgG Enzyme Immunoassay. As shown in Table 3, sensitivity for the detection of IgG antibodies by EIA in ELB ranged from 69 to 31%. For LLB, sensitivity ranged from 92 to 54%. Specificity in the 62 healthy controls ranged from 97 to 85%. For the 128 non-Lyme patient control sera, specificity ranged from 98 to 84%. The two EIAs with a specificity higher than 90% in healthy controls as well as in all non-Lyme patients were the Dako and Genzyme Virotech tests. The presence of EBV, CMV, RF, TP or HIV (but not ANA) contributed to the low specificity of the other EIAs. In Table 4, the positive predictive value in patient population I (LLB sera combined with sera of healthy controls), with an LLB prevalence of 20%, was the highest for the Dako test (86%), with a sensitivity of 77% and a specificity of 97% (Table 3), followed by the Genzyme Virotech test (78%), with a sensitivity of 92% and a specificity of 94% (Table 3). Of all IgG EIAs, the Dako test scored the highest positive predictive value (91%) in population II (LLB sera and sera from non-Lyme patient controls) and population III (94%) (LLB sera combined with sera of non-Lyme patient controls, with EBV and CMV patient controls excluded), both with an LLB prevalence of 20% (Table 4).

Table 4 shows clearly that the low positive predictive value in the EIAs tested is not caused solely by the CMV and EBV sera. Mean index values of positive results in the ELB, LLB and healthy control groups were more than twice the baseline for all EIAs. Positive-reacting TP and HIV sera gave indexes more than twice the baseline for the Behring, Boehringer, Genzyme Virotech and IBL tests. Positive-reacting RF sera gave indexes more than twice the baseline in the Behring, Dako and IBL tests. ANA sera that scored more than twice the baseline were detected with the Genzyme Virotech and IBL IgG EIAs. CMV sera resulting in a positive EIA scored more than twice the baseline in the Behring, IBL and Milenia tests and more than four times the baseline in the Dako test. Positive-reacting EBV sera scored higher than two times the baseline in the Behring, Genzyme Virotech and IBL tests. As shown in Table 5, the intra-assay precision was higher than 90% for all IgG EIAs tested.

Performance of Western Blots. Sensitivity and specificity of the two WBs for the detection of IgM and IgG antibodies for Lyme borreliosis are listed in Tables 2

and 3, respectively. Predictive values for positive and negative results for the detection of IgM antibodies in ELB and IgG antibodies in LLB are listed in Table 4.

IgM Western Blot. Sensitivity varied from 50 to 46% in the 26 ELB patients and from 62 to 54% in the 13 LLB patients (Table 2). In the healthy control group, Genzyme Virotech and MRL WBs showed a specificity of 89% and 98%, respectively. As seen in Table 2, specificity of the test was the highest for the MRL WB in all patient groups except the CMV group. Low specificity caused by EBV and CMV was due to reactivity to OspC. The positive predictive value of IgM WB results in all patient populations was the highest for the MRL WB (Table 4). Excluding EBV and CMV positive sera from the patient control group increased the positive predictive value of the IgM WB results in the MRL WB from 53% up to 85%. (Table 4, population III).

IgG Western Blot. The sensitivity of the IgG Western blots in the ELB group varied form 27 to 4% (Table 3). In the LLB group, sensitivity for the immunoblots was 46% for both WBs.

In the healthy control group, specificity varied from 97 to 82%. In the non-Lyme patient control group, Genzyme Virotech showed a specificity lower than 90% in the CMV, RF and HIV control group. The MRL WB showed a specificity of 100% in all non-Lyme groups. As shown in Table 4, among all IgG WBs, the positive predictive value was highest for MRL WB and was not affected by the non-Lyme patient control group. Excluding the EBV and CMV positive sera from the patient population afforded no apparent increase in the positive predictive value of the Genzyme Virotech IgG WB (Table 4).

IgM Enzyme Immunoassay-Western Blot Two-Test Protocol. The sensitivity and specificity of an EIA-WB two-test protocol for the detection of IgM antibodies in ELB and LLB sera is presented in Table 6. Predictive values for positive and negative results of the EIA-WB two-test protocol for the detection of IgM antibodies in ELB are listed in Table 7. As seen in Table 6, the IgM EIA-WB two-test combination with the highest sensitivity for detecting ELB was the Genzyme Virotech EIA and WB combination (50%). The specificity of the most sensitive two-test combination was 100% for the healthy control group and 97% for the non-Lyme patient control group when EBV and CMV were excluded. The second best in sensitivity was the Behring EIA in combination with either the Genzyme Virotech or the MRL WB, the IBL EIA with the MRL WB, and the Genzyme Virotech EIA with the MRL WB. Specificity for the second best tests in sensitivity was 100% for the healthy control group and 99% or 100% for the non-Lyme patient control group when EBV and CMV patients were excluded. In Table 7, the positive predictive value of IgM results in the two-test protocol for population I (ELB + healthy controls) with a prevalence of 20% ELB patients was 100% for all test combinations except for the IBL EIA-Genzyme Virotech WB (74%) and the Milenia EIA-MRL or Genzyme Virotech WB test combination (25% and 38%, respectively). If CMV- and EBV-positive patients were excluded from the patient population, the positive predictive value of the results was 100% for all EIA-

Table 6 Comparison of sensitivity and specificity of enzyme immunoassay (EIA) – Western blot (WB) two-test protocol combinations for the detection of IgM/IgG antibodies for Lyme borreliosis

		Percent sensitivity				Percent specificity					
		ELB ($n=26$)		LLB ($n=13$)		Healthy controls ($n=62$)		Non-Lyme ($n=128$)		Non-Lyme without EBV/CMV ($n=92$)	
Test		IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
Behring EIA	+GV WB	46	23	38	46	100	94	92	99	99	99
	+MRL WB	46	4	46	38	100	97	95	100	100	100
Boehringer EIA	+GV WB	31	15	31	46	100	94	93	98	100	98
	+MRL WB	35	4	46	38	100	97	94	100	100	100
Dako EIA	+GV WB	35	19	38	38	100	97	95	99	100	99
	+MRL WB	42	4	46	38	100	97	96	100	100	100
Genzyme Virotech EIA	+GV WB	50	19	38	38	100	95	88	99	97	99
	+MRL WB	46	4	46	38	100	97	94	100	100	100
IBL EIA	+GV WB	35	15	31	31	97	94	88	97	97	97
	+MRL WB	46	4	38	31	100	97	92	100	100	100
Milenia EIA[a]	+GV WB	12	12	46	46	95	95	99	99	99	99
	+MRL WB	4	4	46	46	97	97	100	100	100	100

[a] IgM+IgG

GV WB, Genzyme Virotech Western blot; MRL WB, MRL Western blot; ELB, early Lyme borreliosis; LLB, late Lyme borreliosis; EBV, Epstein-Barr virus; CMV, cytomegalovirus

Table 7 Comparison of positive and negative predictive values of enzyme immunoassay (EIA) – Western blot two-test protocol combinations for the detection of IgM/IgG antibodies for Lyme borreliosis. Predictive value is calculated for a prevalence of 20% early Lyme borreliosis and 20% late Lyme borreliosis tested in IgM and IgG populations, respectively

Test		Population I[a]		Population II[b]		Population III[c]	
		PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
IgM test							
Behring EIA	+GV WB	100	88	59	87	92	88
	+MRL WB	100	88	70	88	100	88
Boehringer EIA	+GV WB	100	85	53	84	100	85
	+MRL WB	100	86	59	85	100	86
Dako EIA	+GV WB	100	86	64	85	100	86
	+MRL WB	100	87	72	87	100	87
Genzyme Virotech EIA	+GV WB	100	89	51	88	81	89
	+MRL WB	100	88	66	87	100	88
IBL EIA	+GV WB	74	86	42	84	74	86
	+MRL WB	100	88	59	87	100	88
Milenia EIA[d]	+GV WB	38	81	75	82	75	82
	+MRL WB	25	80	100	81	100	81
IgG test							
Behring EIA	+GV WB	66	87	92	88	92	88
	+MRL WB	76	86	100	87	100	87
Boehringer EIA	+GV WB	66	87	85	88	85	88
	+MRL WB	76	86	100	87	100	87
Dako EIA	+GV WB	76	86	90	86	90	86
	+MRL WB	76	86	100	87	100	87
Genzyme Virotech EIA	+GV WB	66	86	90	86	90	86
	+MRL WB	76	86	100	87	100	87
IBL EIA	+GV WB	56	84	72	85	72	85
	+MRL WB	72	85	100	85	100	85
Milenia EIA[d]	+GV WB	70	88	92	88	92	88
	+MRL WB	79	88	100	88	100	88

[a] Lyme borreliosis and healthy control group
[b] Lyme borreliosis and non-Lyme patient control group
[c] Lyme borreliosis and non-Lyme patient control group without cytomegalovirus and Epstein-Barr virus patient controls
[d] IgM + IgG
GV WB, Genzyme Virotech Western blot; MRL WB, MRL Western blot; PPV, positive predictive value; NPV, negative predictive value

MRL WB test combinations. The Genzyme EIA-WB test combination gave a positive predictive value for IgM results of 81% when EBV and CMV patients were excluded from the non-Lyme patient controls. The Boehringer and the Dako EIAs in combination with Genzyme Virotech or MRL WB both gave a positive predictive value of 100% for IgM results when EBV and CMV patients were excluded.

The EIA and WB two-test combinations with the highest sensitivity, specificity and positive predictive values for IgM results are the Behring, Dako, Genzyme Virotech or IBL EIA in combination with the MRL WB.

IgG Enzyme Immunoassay-Western Blot Two-Test Protocol. As shown in Table 6, a maximum sensitivity of 46% for the detection of LLB was found with the Behring or Boehringer EIA in combination with the Genzyme Virotech WB and with the Milenia EIA in combination with the Genzyme Virotech or the MRL WB. Specificity in the healthy control group and non-Lyme patients controls ranged from 94 to 100% for all test combinations. As shown in Table 7, the Milenia EIA – MRL WB test combination, with a sensitivity of 46%, gave the maximum positive predictive value (79%) for IgG results in the two-test protocol for population I (LLB + healthy control group) with an LLB prevalence of 20%. The Behring, Boehringer, Dako and Genzyme Virotech EIA in combination with MRL WB gave for the same population a positive predictive value of 76% for IgG results. The Dako EIA showed no difference for population I in the positive predictive value of IgG results (76%) for both WBs used in the two-test combination. Exclusion of EBV and CMV from the patient control group did not increase the predictive value of all two-test combinations.

Discussion

Of the 11 EIAs tested, the Behring and Genzyme Virotech IgM tests were superior to the others with respect to sensitivity, specificity and predictive value of detecting ELB when EBV and CMV patients were excluded but showed unacceptably low positive predictive values for the IgG test results. The Dako IgG test, which yielded a lower sensitivity in detecting LLB, showed the highest positive predictive value in a population of LLB patients that included either healthy

controls or non-Lyme patient controls. Overall, low specificity in IgM EIA was mainly observed in the EBV IgM- and CMV IgM-positive sera group.

The derived index values showed clearly that sera of EBV and CMV patients who reacted positively in EIA were significantly higher than the baseline. Because laboratory testing for LB is mainly used to confirm the clinical diagnosis, the sensitivity in combination with the predictive values of positive and negative results are the most important. Accordingly, we compared in this study the results in populations consisting of LB patients with healthy controls and non-Lyme patients. In contrast to most studies, the non-Lyme patient group contained sera positive for EBV, CMV, RF, ANA, TP and HIV. Results indicated that EBV and CMV are the main causes of false-positive reactions in IgM EIAs and can only be discriminated from ELB with an additional test for EBV and CMV. This was clearly shown by the finding that the specificity and positive predictive value in population II increased substantially after exclusion of the CMV- and EBV-positive sera. The predictive value of positive results in a patient population consisting of LLB either with healthy controls or non-Lyme patient controls was lower than 80% for all IgG EIAs except for the Dako IgG EIA. However, 5–10% of asymptomatic seropositive LB cases are commonly found in a healthy population and have also been reported from the Netherlands [27, 37].

For WB, the observer-independent interpretation helped considerably in the quantification of faint bands that posed interpretation difficulties on visual examination. Compared to the visual scoring, our quantitative approach scored a higher specificity without losing sensitivity (data not presented). Instead of using band values expressed as percentages of a fixed reference band performed by Pachner et al. [38], we used a correction method that could provide additional information on inter- and intra-blot variation.

The reason for the low sensitivity of IgM and IgG WB was probably due to very weak bands or the total lack of specific bands. For the Genzyme Virotech IgG WB, specificity in healthy controls increased from 82 to 97%, without loss of sensitivity, when OspA or OspB was excluded from the criteria for a positive blot interpretation, as recommended by Hauser et al. [29] for WBs using *Borrelia burgdorferi* sensu stricto as antigen. As indicated by others [39], antibodies against OspA and OspB proteins were rarely detected in LLB patients. Although the detection of antibodies against the OspA and OspB proteins may not be crucial for the diagnosis in terms of sensitivity, Leung et al. [40] indicated these proteins to be very specific markers of LLB when they are detected in tandem.

In our study, reactions to both OspA and OspB were only seen in 15% of the LLB. A very low specificity was found when we used the criteria resulting in a positive IgG blot with at least one band of p83, p58, p56, OspC, p21 or p17, as recommended by Hauser et al. [29] for blots using the sensu stricto strain. This was caused by the appearance of the p56 band in the control group. In the MRL WB the criteria recommended by Hauser et al. [29] for WBs using *Borrelia garinii* as antigen gave a lower specificity compared to the criteria of the manufacturer. The recommended criteria for IgM blots, where a strong reaction to p41 (flagellin) increases sensitivity without significant loss of specificity, could not be confirmed in our study. In all control groups, cross-reactive IgM responses, particularly with the 41 kDa flagellar antigen, occurred more frequently in the Genzyme Virotech WB compared to the MRL WB but did not affect the evaluation when the manufacturer's criteria were strictly followed. Low specificity in IgM WB was mainly observed in the EBV- and CMV-positive sera. In these sera, WB showed a predominant reaction with the highly specific OspC, which hampered differentiation of these non-Lyme sera by WB. Changing the criteria for positive IgM WB interpretation in the MRL WB to two bands of 41 kDa, 39 kDa or 23 kDa (OspC) improved neither sensitivity nor specificity.

Several technical problems of Western blotting using sonicated *Borrelia burgdorferi* cells have been mentioned in the literature [31]. Still, information about data such as (a) strain-dependent molecular weights of similar / identical proteins of the spirochete; (b) comigration of multiple proteins to the same area; (c) use of monoclonal antibodies for protein identification and (d) 2-D mapping, which might indicate a comigration of different proteins, are often not provided by the manufacturers. Therefore, a false-positive reaction in Lyme Western blotting for EBV- and CMV-positive sera caused by an OspC co-migrating protein cannot be ruled out.

The findings of Hauser et al. [29] that the use of a WB with a European strain gave a higher sensitivity for a European serum panel than the WB with an American isolate for detecting early or late Lyme borreliosis in, respectively, IgM and IgG was not confirmed by our results. Although the use of a European strain in WB showed a higher positive predictive value, specificity of the WB in RF-, TP- and HIV-positive sera was lower when an American strain was used. The cross-reaction with *Borrelia burgdorferi* of sera positive for TP or HIV has been reported [41], but we had no trouble differentiating such sera from the sera of patients with LB in the WB test. Preabsorption with RF and *Reiter* antigens to improve specificity was not performed in our WB testing as these were not standard antigens included in the test kits. However, in our study, preabsorption would not have affected the overall performance of the WB kits. The observed percentages of positive reactions in the healthy control group of 3% for

the MRL and the Genzyme Virotech IgG WBs (when excluding OspA and OspB in the interpretation criteria from the Genzyme Virotech WB) are of the same size as of the 5% asymptomatic healthy controls found positive [27, 37] in a Dutch population and are therefore most likely a result of previous asymptomatic infections. In contrast to the findings of Dressler et al. [31] and the recommendations of ASTPHLD and CDC to use a two-step protocol where WB is used to increase specificity, we found that, with the IgM EIAs tested, performing an additional EBV and, if indicated, a CMV test is a more effective way of increasing the specificity without a significant loss in sensitivity in case of ELB.

This study showed clearly that not only sera of healthy controls but, preferably, sera of patients with a similar differential diagnosis as LB should be used for the evaluation of the serodiagnostic tests for LB. As positive and negative predictive values are the most relevant parameters for clinical decision making, not only sensitivity and specificity but also the predictive values for positive and negative results should be compared. The two tests presenting the best results for detecting ELB in a population without EBV and CMV patients were the Behring IgM EIA followed by the Genzyme Virotech IgM EIA. Despite its relatively low sensitivity in detecting LLB, the positive predictive value in the populations studied was the highest for the Dako IgG EIA. It should be realized, however, that a specificity and positive predictive value of 100% can never be expected because of possible previous asymptomatic infections that can result in a positive result in LB serological tests. The use of a WB test as a confirmatory test gave some improvement over IgG EIA results alone, but for positive IgM EIA results in a geographical region where the LB prevalence is 20% or lower, the exclusion of an EBV infection and, if indicated, a CMV infection, seems more advisable.

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