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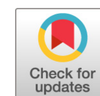

Diagnostic Performance of the Novel BioPlex Lyme Serological Assays in European Patients with Lyme Disease

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ABSTRACT Serodiagnosis of Lyme borreliosis (LB) comes with several drawbacks, among which is limited sensitivity in early disease. This study assesses the sensitivity and specificity of the novel BioPlex 2200 Lyme IgG and Lyme IgM assays. It also assesses potential improvements to the assays through receiver-operating characteristic (ROC) analysis. The BioPlex assays were performed on sera of 158 Dutch patients with physician-confirmed LB (both early localized and disseminated), 800 healthy blood donors from the Netherlands, and 90 cross-reactive controls. The BioPlex (Biopl) assays were compared with two commercial enzyme immunoassays (Euroimmun [Eur]/C6-ELISA) and one immunoblot (recomLine). The highest sensitivity in early LB was achieved with the BioPlex assays, which outperformed the Euroimmun and C6-ELISA (Biopl: 81/88, 92.1%; Eur: 64/88, 72.7%; C6: 72/88, 81.8%). Sensitivity of all assays was comparable in patients with disseminated LB. The BioPlex assays were outperformed in terms of specificity (all healthy blood donors, Biopl: 571/800, 71.4%; Eur: 711/800, 88.9%; C6: 727/800, 90.9%), but further analyses showed promising avenues following cutoff optimization. ROC analysis showed that 2/6 antigens of the combined BioPlex IgG and IgM assays had significantly higher areas under the curve (AUCs) than those of the other analyses. Potential modified versions of the assays based on these antigens largely outperformed the Euroimmun and C6-ELISA in EM patients (Biopl: 81/80, 92.1%) while maintaining a comparable or even higher specificity (Biopl: 714/800, 89.3%). The BioPlex 2200 Lyme IgG and Lyme IgM assays are promising tools for the serodiagnosis of early LB, with the potential to be used as a standalone test. Further research is necessary to validate the findings of this discovery cohort.

KEYWORDS BioPlex Lyme, C6-ELISA, Lyme borreliosis, multiplex flow immunoassay, serology

The most common diagnostic tests for Lyme disease (Lyme borreliosis, LB) are serological assays. Due to variation in *Borrelia burgdorferi sensu lato* genospecies, serological tests are usually specific to either Europe or North America. Guidelines recommend performing serodiagnosis of LB using two-tier testing (1–4). Traditionally, this consists of a highly sensitive enzyme immunoassay (EIA), followed by a more specific immunoblot to confirm all positive or equivocal EIA results. Unfortunately, this standard two-tier testing algorithm using an immunoblot (STTT) has a sensitivity as low as 50% for early LB, specifically for an erythema migrans (EM) (5). For this reason, guidelines have conventionally recommended against serological testing for patients suspected of an EM, even though testing may sometimes be desirable (e.g., for atypical lesions or patients suspected of early LB with nonspecific symptoms only) (1–3).

Recently, various researchers have proposed replacing the immunoblot with a 2nd confirmatory EIA. This so-called modified two-tier testing algorithm (MTTT) using the C6-enzyme-linked immunosorbent assay (ELISA) by Immunetics in the 2nd tier

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was found to have better sensitivity than that of STTT without any significant loss of specificity (6–10).

In the current manuscript, we present another potential method for improving the sensitivity of serological testing for early LB by investigating the diagnostic parameters of the BioPlex 2200 Lyme IgG and Lyme IgM assays (Bio-Rad Laboratories, Hercules, CA, USA). Previous research showed that an assay from the United States based on the same platform may be a promising diagnostic tool, with the potential to function as a standalone serological test with an observed sensitivity and specificity of >90% (11).

In this multiple-gate case-control study (12), we have assessed the BioPlex assays' diagnostic parameters in Dutch patients with EM and disseminated LB, in healthy blood donors (HBDs) from the Netherlands, and in a set of cross-reactive/diseased controls. We have compared them to the diagnostic parameters of two conventional EIAs in various algorithms (STTT, MTTT, IgG only).

MATERIALS AND METHODS

LB sera. Leftover sera from 158 physician-confirmed LB patients were used as cases. These were selected as a convenience sample from the serum bank of the Amsterdam Multidisciplinary Lyme borreliosis Centre, a tertiary referral center for LB in the Netherlands. Selected patients were included in the serum bank between October 1986 and August 2018. Selected sera were divided into three time points: acute, for sera acquired less than 6 weeks after onset of symptoms (EM: $n = 27$, disseminated LB: $n = 7$), early convalescent, for sera acquired 6 to 12 weeks after onset of symptoms (EM: $n = 25$, disseminated LB: $n = 11$), and late convalescent, for sera acquired 12 or more weeks after onset of symptoms (EM: $n = 54$, disseminated LB: $n = 60$). If more than 1 serum from the same patient was available for a given time point, only the first serum from that time point was included. Analyses were performed on a final selection of 190 sera from cases. This included 4 sera from EM patients and 2 sera from disseminated LB patients for whom the onset of symptoms and hence the time point was unknown. These were included in the overall analysis of combined sensitivity but not in any specific time point.

Clinical data were collected by retrospective assessment of patient records (Table 1). LB diagnoses were classified as proven when PCR- or culture-confirmed or as well-defined when based on a composite of clinical features and laboratory work-up as determined by the treating physician (13, 14). In this context, clinical features denote that there were objective unambiguous clinical findings of early or disseminated LB (e.g., expanding erythematous skin lesion of >5 cm, classical Bannwarth syndrome, mono- or oligo-arthritis involving large joints such as the knee); laboratory work-up means specific LB-related work-up (e.g., electrocardiogram [EKG] for Lyme carditis or cerebral spinal fluid [CSF] analysis for Lyme neuroborreliosis) and importantly also encompassed exclusion of other potential causes. For study purposes, the classification of cases was verified by J. W. Hovius (professor of vector-borne infectious diseases) and J. Ursinus (MD-PhD student specializing in LB), based on current European guidelines (15).

Control sera. As healthy controls, sera from adult HBDs were acquired from two different sources. Six hundred sera were acquired from Sanquin, the legally mandated organization for blood donation in the Netherlands. HBDs were selected to provide an even spread of sex, age category, and geographical location across the Netherlands. Sera were selected irrespective of the presence of antibodies against *Borrelia* spp. or other tick-borne pathogens. Sera were donated between October 2018 and March 2019. An additional 200 HBD sera were procured from Cantor BioConnect (Santee, CA, USA). These sera were also obtained from donors from the Netherlands and were donated in September 2018. The median age of all 800 HBDs was 41.1 years (interquartile range [IQR] 33.2 to 58.4); 52.8% were female.

In addition, we included 90 sera from cross-reactive/diseased controls from the CDC Lyme Serum Repository (LSR) (16). These were sera from patients with an Epstein-Barr virus (EBV) infection (infectious mononucleosis, $n = 15$), syphilis ($n = 15$), multiple sclerosis ($n = 15$), fibromyalgia ($n = 15$), rheumatoid arthritis ($n = 15$), and severe periodontitis ($n = 15$).

BioPlex 2200 Lyme IgG and Lyme IgM assays. The BioPlex 2200 Lyme IgG and Lyme IgM assays are fully automated bead-based multiplex flow immunoassays. Antigens were selected from a panel of synthetic peptides and recombinant antigens derived from various *Borrelia burgdorferi sensu lato* genospecies specific to Europe, essentially as previously described (17), and were screened for both IgG and IgM reactivity in another European cohort of clinically characterized acute LB patients and HBDs. The current BioPlex IgG assay contains three antigens: recombinant p58, recombinant DbpA, and synthetic fusion protein FVlsE containing sequences from FlaB (amino acids [aa] 211 to 223) and a modified VlsE (aa 275 to 291). The current BioPlex IgM assay contains three native protein antigens: OspCA, OspCB, and VlsE.

A detailed description of the assays' principle and a comparable procedure have been previously published (17). Briefly, *Borrelia*-specific antibodies in serum were captured onto antigen-coated beads. After washing, the beads were incubated with either anti-human IgM or anti-human IgG antibody-phycoerythrin conjugate. Following additional washes, the beads passed through the detector for the measurement of fluorescent signal generated by bound conjugates. The resulting multiplexed bead signals were independently calibrated for each antigen and reported as an antibody index (AI), defined as the ratio of the experimental signal intensity to that of a calibrator. For each analyte, this calibrator's signal intensity was set at the 98th percentile of the aforementioned different cohort of HBD samples, giving

TABLE 1 Baseline characteristics

Characteristic	LB patient data ($n = 158$)
Female sex, no. of patients (%)	89 (56.3)
Age, median yr (interquartile range)	50.8 (36–60)
First sample collected before antibiotic treatment, no. of patients (%)[a]	124 (78.5)
Early localized LB	
Erythema migrans, no. of patients (%)	88 (55.7)
Classification, no. of patients (%)	
Culture- and/or PCR-positive	60 (68.2)
Well-defined	28 (31.8)
Duration of manifestation (wks)	
Mean (range)[b]	12 (0–38)
Disseminated manifestations, no. of patients (%)	
Acrodermatitis chronica atrophicans	26 (16.5)
Lyme neuroborreliosis	24 (15.2)
Lyme arthritis	7 (4.4)
Multiple disseminated manifestations[c]	4 (2.5)
Unspecified disseminated LB	4 (2.5)
Multiple EM	3 (1.9)
Borrelial lymphocytoma	1 (0.6)
Lyme carditis	1 (0.6)
Classification, no. of patients (%)	
Culture- and/or PCR-positive	19 (27.1)
Well-defined	51 (72.9)
Duration of manifestation (wks)	
Mean (range)[d]	108 (4–894)

[a]Unknown in 6 patients.
[b]Date of onset of manifestation unknown in 4 patients.
[c]Two patients diagnosed with acrodermatitis chronica atrophicans were simultaneously diagnosed with Lyme neuroborreliosis and another was simultaneously diagnosed with Lyme arthritis. One patient was diagnosed with both multiple EM and Lyme arthritis.
[d]Date of onset manifestation unknown in 2 patients.

an AI value of 1.0. An equivocal range of ±10% was then set around the cutoff value. This yields a developer-defined interpretation of a positive result as an AI value of >1.1, a negative result as an AI value of <0.9, and an equivocal result as an AI value of 0.9 to 1.1. Reactivity in one antigen per assay (i.e., p58, DbpA, or FVlsE for IgG; OSpCA, OspcB, or VlsE for IgM) is sufficient to yield a result as positive/equivocal for that assay. Rules for the combination of the IgM/IgG assays into an overall test result are given in the paragraph below, together with interpretation rules for the comparator tests.

Study execution. Apart from the BioPlex assays, two commercially available immunoassays were performed on all LB and HBD samples: the anti-*Borrelia* ELISA (Euroimmun, Lübeck, Germany) and the C6-ELISA (Immunetics/Oxford Immunotec, Oxford, United Kingdom). All sera with an equivocal or positive result in at least one of the EIAs or in the BioPlex assays were tested by the recomLine *Borrelia* IgM and IgG immunoblot (Mikrogen, Neuried, Germany). The BioPlex assays and the Euroimmun were performed at Bio-Rad's facilities; the C6-ELISA and immunoblots were performed at Amsterdam UMC's laboratories. All assays were nonblinded performed according to the developer's/manufacturers' instructions and using prescribed cutoffs. For cross-reactive/diseased control sera, only the BioPlex assays and C6-ELISA were available.

Sera were stored at −80°C and an effort was made to keep thaw-refreeze effects to a minimum. Bands on the immunoblots were interpreted by two independent assessors and also by a third assessor in case of disagreement.

All tests which had a separate IgM and IgG component were interpreted as follows. When either one component or both components were positive, the test was interpreted as positive. When no component was reactive, the test was interpreted as negative. When one component was equivocal and the other negative, or when both components were equivocal, the test was interpreted as equivocal. Apart from analyzing first-tier assays as standalone tests, assays were combined in an STTT algorithm (commercial EIA/BioPlex followed by immunoblot) and an MTTT algorithm (Euroimmun/BioPlex followed by the C6-ELISA). In both, equivocal first-tier results were classified as positive, while equivocal second-tier results were classified as negative. In single-tier algorithms, equivocal results were interpreted as positive.

Statistical analysis. The 95% confidence intervals of the sensitivity and specificity were determined using Clopper-Pearson. Diagnostic parameters of tests within each serum group were compared to one another using the (exact) McNemar test. Subanalyses to compare diagnostic parameters between independent groups (EM versus disseminated) were performed using Fisher's exact test. In the receiver-operating characteristic (ROC) analysis, areas under the curve (AUCs) were compared using a z-test. For all analyses, P values of <0.05 were considered statistically significant.

Ethical statement. The study was conducted according to the principles of the Declaration of Helsinki and in conformity with institutional regulations and guidelines. Since deidentified leftover

TABLE 2 Sensitivity of all algorithms in EM patients[a,b]

Algorithm	Acute ($n = 27$)		Early convalescent ($n = 25$)		Late convalescent ($n = 54$)		All time points combined ($n = 88$)	
	No. true positive (sensitivity %)	95% CI	No. true positive (sensitivity %)	95% CI	No. true positive (sensitivity %)	95% CI	No. true positive (sensitivity %)	95% CI
Biopl-IgM	12 (44.4)	25.5–64.7	15 (60.0)	38.7–78.9	29 (53.7)	39.6–67.4	48 (54.5)	43.6–65.2
Biopl-IgG	18 (66.7)	46.0–83.5	22 (88.0)	68.8–97.5	49 (90.7)	79.7–96.9	78 (88.6)	80.1–94.4
Eur-IgM	7 (25.9)	11.1–46.3	10 (40.0)	21.1–61.3	19 (35.2)[c]	22.7–49.4	32 (36.4)[c]	26.4–47.3
Eur-IgG	9 (33.3)[c]	16.5–54.0	12 (48.0)[c]	27.8–68.7	36 (66.7)[c]	52.5–78.9	54 (61.4)[c]	50.4–71.6
Single: Biopl-total	20 (74.1)	53.7–88.9	24 (96.0)	79.7–99.9	52 (96.3)	87.3–99.6	81 (92.1)	84.3–96.7
Single: Eur-total	12 (44.4)[c]	25.5–64.7	18 (72.0)[c]	50.6–87.9	41 (75.9)[c]	62.4–86.5	64 (72.7)[c]	62.2–81.7
Single: C6	16 (59.3)	38.8–77.6	22 (88.0)	78.8–97.5	44 (81.5)[c]	68.6–90.8	72 (81.8)[c]	72.2–89.2
STTT: Biopl/blot	7 (25.9)	11.1–46.3	7 (28.0)	12.1–49.4	19 (35.2)	22.7–49.4	28 (31.8)	22.2–42.6
STTT: Eur/blot	7 (25.9)	11.1–46.3	7 (28.0)	12.1–49.4	19 (35.2)	22.7–49.4	28 (31.8)	22.2–42.6
STTT: C6/blot	6 (22.2)	8.6–42.3	6 (24.0)	9.4–45.1	18 (33.3)	21.1–47.5	27 (30.7)	21.3–41.4
MTTT: Biopl/C6	15 (55.6)	35.3–74.5	20 (80.0)	59.3–93.2	44 (81.5)	68.6–90.8	70 (79.6)[e]	69.6–87.4
MTTT: Eur/C6	11 (40.7)[e]	22.3–61.2	15 (60.0)[e]	38.7–78.9	38 (70.4)[d,e]	56.4–82.0	60 (68.2)[d,e]	57.4–77.7
Biopl-IgG only	18 (66.7)	46.0–83.5	22 (88.0)	78.8–97.5	49 (90.7)	79.7–96.9	78 (88.6)	80.1–94.4

[a]A test result was considered to be (true) positive when the IgM component, IgG component, or both were positive. Equivocal first-tier EIA results were interpreted as positive. For the combined sensitivity of all time points, a patient was scored positive if one or more time points were positive.
[b]Abbreviations/explanations: single, single-tier; STTT, standard two-tier testing; MTTT, modified two-tier testing; 95% CI, 95% confidence interval; Biopl, BioPlex; Eur, Euroimmun; C6, C6-ELISA; blot, recomLine immunoblot.
[c]$P < 0.05$ compared to Biopl-IgM/IgG (equivalent component) or Biopl-total (as single-tier). STTT algorithms were comparable throughout.
[d]$P < 0.05$ compared to Biopl/C6 (for MTTT).
[e]$P < 0.05$ compared to Biopl-IgG only.

patient samples were utilized, the Dutch Medical Research Involving Human Subjects Act does not apply to this study and no informed consent was asked from LB patients. HBDs from Sanquin and Cantor BioConnect provided explicit written consent for use of their deidentified leftover materials for research purposes. Informed consent from cross-reactive/diseased controls was acquired at the time of inclusion in the CDC LSR (16).

RESULTS

Diagnostic parameters for the BioPlex 2200 Lyme IgG and Lyme IgM assays separately and in combination (hereafter: BioPlex combined) are shown in Tables 2 and 3 and Table S1, together with the diagnostic parameters of the other first-tier assays. Results are shown in both single-tier and STTT algorithms. The overall highest sensitivity in EM was generated by the BioPlex combined (Biopl). Comparing the combined sensitivity of all time points for EM patients, the BioPlex combined as a standalone test performed significantly better than the other first-tier assays (Biopl versus Euroimmun [Eur]: $P < 0.001$, Biopl versus C6: $P = 0.012$). The BioPlex combined also demonstrated the highest sensitivity for each time point in EM, but these differences were not always statistically significant. In an STTT algorithm, sensitivities were comparable between the first-tier assays. This was also the case for disseminated LB, in which the BioPlex combined performed on par with the other first-tier assays. Sensitivities of all assays were also comparable between proven and well-defined cases, in both EM and disseminated LB patients. For both groups of HBDs individually and combined, the BioPlex combined demonstrated significantly lower specificity than did the other assays (Biopl versus C6 and Biopl versus Eur: $P < 0.01$). In the STTT algorithm, this difference was still apparent for the comparison with the C6/blot in the Sanquin HBDs and combined sets of HBDs (for both, Biopl/blot versus C6/blot: $P < 0.01$), although now specificity of the Biopl/blot was well above 90%.

Thus, the BioPlex combined demonstrated higher sensitivity but lower specificity than did the commercial EIAs. Therefore, we investigated various strategies to ameliorate this common problem of tradeoff between sensitivity and specificity. In line with previous studies, we have sought to improve specificity without significant or clinically relevant loss of sensitivity by assessing the BioPlex in an MTTT algorithm or by looking at its IgG assay only (6–10, 18).

TABLE 3 Specificity of all algorithms[a,b,c]

Algorithm	Sanquin HBDs ($n = 600$)		Other HBDs ($n = 200$)		All HBDs ($n = 800$)	
	No. true negative (specificity %)	95% CI	No. true negative (specificity %)	95% CI	No. true negative (specificity %)	95% CI
Biopl-IgM	460 (76.7)	73.1–80.0	159 (79.5)	73.2–84.9	619 (77.4)	74.3–80.2
Biopl-IgG	548 (91.3)	88.8–93.5	180 (90.0)	85.0–93.8	728 (91.0)	88.8–92.9
Eur-IgM	588 (96.2)[d]	96.5–99.0	186 (93.0)[d]	88.5–96.1	763 (95.4)[d]	93.7–96.7
Eur-IgG	559 (93.2)[d]	90.8–95.1	180 (90.0)	85.0–93.8	739 (92.4)	90.3–94.1
Single: Biopl-total	424 (70.7)	66.8–74.3	147 (73.5)	66.8–79.5	571 (71.4)	68.1–74.5
Single: Eur-total	543 (90.5)[d]	87.9–92.7	168 (84.0)[d]	78.2–88.8	711 (88.9)[d]	86.5–91.0
Single: C6	548 (91.3)[d]	88.8–93.5	179 (89.5)[d]	84.4–93.4	727 (90.9)[d]	88.6–92.8
STTT: Biopl/blot	566 (94.3)	92.2–96.0	189 (94.5)	90.4–97.2	755 (94.4)	92.5–95.9
STTT: Eur/blot	571 (95.2)	93.1–96.7	190 (95.0)	91.0–97.6	761 (95.1)	93.4–96.5
STTT: C6/blot	579 (96.5)[d]	94.7–97.8	194 (97.0)	93.6–98.9	773 (96.6)[d]	95.1–97.8
MTTT: Biopl/C6	568 (94.7)[e]	92.6–96.3	187 (93.5)	89.1–96.5	755 (94.4)[e]	92.5–95.9
MTTT: Eur/C6	573 (95.5)[e]	93.5–97.0	189 (94.5)[e]	90.4–97.2	762 (95.3)[e]	93.5–96.6
Biopl-IgG only	548 (91.3)	88.8–93.5	180 (90.0)	85.0–93.8	728 (91.0)	88.8–92.9

[a]Specificities of MTTT algorithms were comparable throughout HBD groups.
[b]A test result was considered to be (true) negative when neither the IgM component nor the IgG component was positive. Equivocal first-tier EIA results were interpreted as positive.
[c]Abbreviations/explanations: single, single-tier; STTT, standard two-tier testing; MTTT, modified two-tier testing; 95% CI, 95% confidence interval; HBD, healthy blood donor; Biopl, BioPlex; Eur, Euroimmun; C6, C6-ELISA; blot, recomLine immunoblot.
[d]$P < 0.05$ compared to Biopl-IgM/IgG (equivalent component), Biopl-total (as single-tier), or Biopl/blot (for STTT).
[e]$P < 0.05$ compared to Biopl-IgG only.

Comparisons between assays in the MTTT algorithm showed that the BioPlex/C6 combination had consistently higher sensitivity than that of the Euroimmun/C6 combination in EM. This rose only to the level of statistical significance in late-convalescent-phase sera (Biopl/C6 versus Eur/C6: $P = 0.031$) and for all time points combined (Biopl/C6 versus Eur/C6: $P = 0.002$). This increase in sensitivity in EM for the BioPlex/C6 combination did not come at the cost of a decrease in specificity in HBDs, as these were comparable with those of the Euroimmun/C6 combination (all comparisons: $P > 0.05$).

Based on the reactivity of the individual components (i.e., IgM/IgG) of the BioPlex and Euroimmmun assays, we hypothesized that dropping the BioPlex IgM assay would yield a significant improvement to the overall false-positivity rate with only a marginal effect on sensitivity (Fig. S1). Doing so, we confirmed that the BioPlex IgG assay maintained a higher sensitivity in EM than did the Eur/C6 algorithm for each time point (acute and early convalescent: $P = 0.016$, late convalescent: $P = 0.001$) and either MTTT variant for the combination of all time points (Biopl-IgG versus Biopl/C6: $P = 0.008$; Biopl-IgG versus Eur/C6: $P < 0.001$). However, observed specificity was still lower than that of both MTTT algorithms for most comparisons (e.g., all HBDs: Biopl-IgG versus Biopl/C6 $P < 0.001$).

In cross-reactive/diseased controls, we observed a picture similar to that with the HBDs (Table 4). Specificity of the BioPlex combined was lower than that of the C6-ELISA ($P < 0.001$), driven for a large part by the BioPlex IgM assay's reactivity in sera from EBV and syphilis patients. Similarly, this could be ameliorated by using it in an MTTT algorithm or by using the IgG assay only (both options versus C6: $P > 0.05$).

Next, we investigated whether the BioPlex's prescribed cutoffs were adequate. Initial analyses confirmed that there was a considerable false-positivity rate in HBDs for the BioPlex IgM assay (Fig. S2a to f). Of all antigens, the VlsE antigen in the IgM assay had the greatest reactivity in HBDs ($n = 109/800$, 13.6%), while the other antigens had a false-positivity rate ranging from 4.6% (OspCA) to 7.4% (p58).

Therefore, we performed ROC analyses to assess the discriminatory value of each antigen in both BioPlex kits (Fig. S3) and to optimize the cutoff for all antigens with a sufficient AUC, which we defined as an AUC value of >0.8 (i.e., excellent or good). With serodiagnosis of EM in mind, sera from disseminated LB patients were excluded.

TABLE 4 Specificity of selected assays/algorithms in cross-reactive/diseased controls[a,b]

Algorithm	Fibromyalgia ($n = 15$) No. true neg. (spec %)	EBV ($n = 15$) No. true neg. (spec %)	MS ($n = 15$) No. true neg. (spec %)	Periodontitis ($n = 15$) No. true neg. (spec %)	RA ($n = 15$) No. true neg. (spec %)	Syphilis ($n = 15$) No. true neg. (spec %)	Combined ($n = 90$) No. true neg. (spec %)	Combined ($n = 90$) 95% CI
Biopl-IgM	15 (100)	6 (40.0)	13 (86.7)	13 (86.7)	12 (80.0)	8 (53.3)	67 (74.4)	64.1–83.1
Biopl-IgG	15 (100)	15 (100)	15 (100)	15 (100)	14 (93.3)	14 (93.3)	88 (97.8)	92.2–99.7
Single: Biopl-total	15 (100)	6 (40.0)	13 (86.7)	13 (86.7)	12 (80.0)	7 (46.7)	66 (73.3)	63.0–82.1
Single: C6	15 (100)	13 (86.7)	15 (100)	15 (100)	15 (100)	14 (93.3)	87 (96.7)	90.6–99.3
MTTT: Biopl/C6	15 (100)	14 (93.3)	15 (100)	15 (100)	15 (100)	15 (100)	89 (98.9)	94.0–100.0
Biopl-IgG only	15 (100)	15 (100)	15 (100)	15 (100)	14 (93.3)	14 (93.3)	88 (97.8)	92.2–99.7

[a]A test result was considered to be (true) positive when either the IgM component, IgG component, or both were positive. Equivocal first-tier EIA results were interpreted as positive.
[b]Abbreviations/explanations: EBV, Epstein-Barr virus infection (infectious mononucleosis); MS, multiple sclerosis; RA, rheumatoid arthritis; single, single-tier; MTTT, modified two-tier testing; 95% CI, 95% confidence interval; Biopl, BioPlex; C6, C6-ELISA; spec, specificity.

Furthermore, we included only the first serum from each EM patient to prevent bias due to repeated sampling. We compared these EM sera ($n = 88$) with sera from all HBDs ($n = 800$). We found only FVlsE and p58 to have an AUC value of >0.8. Both antigens' AUCs were higher than those of all other antigens ($P < 0.001$), which all had either a fair or a poor AUC. FVlsE's AUC was also higher than that of p58 ($P < 0.001$).

After analysis of cutoff optimization, we devised three potential modified versions of the BioPlex assays, which (i) included only FVlsE and p58 using the current cutoffs, (ii) included only FVlsE and p58 at cutoffs resulting in a specificity of >90% for each individual antigen and a sensitivity of approximately 90% in either antigen, or (iii) included only FVlsE at a cutoff resulting in a specificity of >90%. For option 2, the cutoff of FVlsE and p58 was set at AI ≥ 0.3and AI ≥ 0.4, respectively. For option 3, the FVlsE cutoff was set at AI ≥ 0.3 as well. The antigens in option 1 and 2 were combined using the Boolean operator OR to give a final result. These modifications were applied to the original data set, which also included subsequent EM sera from the same patient.

All modified versions of the BioPlex had a marked improvement over the original version in false-positivity rate, resulting in specificities comparable to those of the C6-ELISA in all HBDs (Tables 5 and 6). Modified versions 1 and 3 significantly exceeded Euroimmun's specificity in the Other HBD cohort and in all HBDs combined, resulting in a slight but not significant decrease in sensitivity compared to that of the original BioPlex combined. However, in late convalescent EM and for the combination of all time points, modifications 2 and 3 still significantly outperformed the C6-ELISA, while the Euroimmun was significantly outperformed by all modifications at said time points and by modification 2 at the acute time point.

Comparing the three modifications to one another, all were comparable in terms of sensitivity ($P > 0.05$), but options 1 and 3 were superior to option 2 with respect to specificity ($P < 0.01$) in the other HBDs cohort and all HBDs combined. Furthermore, all options were comparable to one another in the cross-reactive/diseased controls and performed on par with the comparator test (all modified Biopl versus C6: $P > 0.05$). False-positivity was seen only for EBV infection (modified Biopl 2 and 3: 2/15, 13.3%) and syphilis (all modified Biopl: 1/15, 6.7%).

DISCUSSION

In the current study, we have investigated the diagnostic parameters of the novel BioPlex 2200 Lyme IgG and Lyme IgM assays in Dutch LB patients and controls.

As hypothesized, the combined BioPlex assays had a markedly improved sensitivity over that of conventional first-tier EIAs for patients with early localized LB. While this result was not statistically significant for the comparison with the C6-ELISA in acute and early-convalescent-phase samples, we find it likely that this can be ascribed to small sample sizes at those time points. Taking all time points together, the combined

TABLE 5 Diagnostic parameters of modified BioPlex in EM sera[a,b]

Modified BioPlex assay option no.	Acute ($n = 27$)		Early convalescent ($n = 25$)		Late convalescent ($n = 54$)		All time points combined ($n = 88$)	
	No. true positive (sensitivity %)	95% CI	No. true positive (sensitivity %)	95% CI	No. true positive (sensitivity %)	95% CI	No. true positive (sensitivity %)	95% CI
1	17 (63.0)	42.4–80.6	22 (88.0)	78.8–97.5	49 (90.7)[d]	79.7–96.9	78 (88.6)[d]	80.1–94.4
2	19 (70.4)[d]	49.8–86.3	23 (92.0)	74.0–99.0	52 (96.3)[c,d]	87.3–99.6	81 (92.1)[c,d]	84.3–96.7
3	18 (66.7)	46.0–83.5	23 (92.0)	74.0–99.0	52 (96.3)[c,d]	87.3–99.6	81 (92.1)[c,d]	84.3–96.7

[a]Abbreviations: 95% CI, 95% confidence interval; HBD, healthy blood donor; C6, C6-ELISA.
[b]Modified BioPlex assay parameters: option 1, FVlsE + p58 at original cutoffs; option 2, FVlsE + p58 at modified cutoffs; option 3, FVlsE only at modified cutoff.
[c]$P < 0.05$, sensitivity significantly higher than that of Single: C6 (data presented in Table 2); specificities were comparable to those of the C6 throughout.
[d]$P < 0.05$, sensitivity/specificity significantly higher than that of Single: Eur-total (data presented in Tables 2 and 3).

BioPlex assays outperformed both the Euroimmun assay and C6-ELISA in terms of sensitivity in EM. The observed reactivity of these conventional first-tier assays in EM sera was largely comparable to that of previously reported diagnostic parameters (10, 19–21) and should therefore be considered representative. All assays performed comparably in disseminated LB patients. Unfortunately, this increase in sensitivity of the BioPlex assays did come at a cost of a reduction in specificity in both HBDs and cross-reactive controls when using the prescribed cutoffs. This was driven primarily by the IgM assay. However, our results show that this problem can be ameliorated by using the BioPlex assay in an MTTT algorithm. The resulting BioPlex/C6 combination outperformed the Euroimmun/C6 combination in terms of overall sensitivity while maintaining comparable specificity.

As the C6-ELISA will be withdrawn from the market, we performed further subanalyses on the BioPlex assays, which suggested that their composition and prescribed cutoffs could be optimized. Due to our limited sample size, we could not use this study for both optimization and subsequent validation. Future research should further determine the best assay composition and cutoffs and validate those in a new European cohort. Based on our data, it seems that the FVlsE and p58 antigens from the IgG assay are the most promising candidates to focus this research on, while the antigens from the IgM assay may require further development in order to achieve acceptable discriminatory value.

Increasing sensitivity of laboratory testing is an important step in improving the diagnostic process for early LB. Despite the scientific consensus that an EM is a clinical diagnosis, it must be noted that it is not always an easy diagnosis to make. EMs can be very atypical and resemble any number of other cutaneous conditions (22–25), resulting in a risk of missing the LB diagnosis and accompanying therapeutic window at a time when therapy is most effective. In rare cases, an EM can be absent, with the only symptoms of early LB being more general symptoms such as fatigue, subfebrile temperature, myalgia, or arthralgia (26). Such patients may benefit from a diagnostic test with a higher sensitivity early in disease, such as the BioPlex assays.

Of course, this should not come at the cost of an unacceptably low specificity. Specificity of the combined BioPlex assays can be brought on par with that of other first-tier assays, either by using it in an MTTT algorithm or by changing their antigen

TABLE 6 Diagnostic parameters of modified BioPlex in control sera[a,b]

Modified BioPlex assay option no.	Sanquin HBDs ($n = 600$)		Other HBDs ($n = 200$)		All HBDs ($n = 800$)		All CRCs ($n = 90$)	
	No. true negative (specificity %)	95% CI	No. true negative (specificity %)	95% CI	No. true negative (specificity %)	95% CI	No. true negative (specificity %)	95% CI
1	551 (91.8)	89.4–93.9	181 (90.5)[c]	85.6–94.2	732 (91.5)[c]	89.4–93.3	89 (98.9)	94.0–100.0
2	536 (89.3)	86.6–91.7	178 (89.0)	83.8–93.0	714 (89.3)	86.9–91.3	87 (96.7)	90.6–99.3
3	551 (91.8)	89.4–93.9	183 (91.5)[c]	86.7–95.0	734 (91.8)[c]	89.6–93.6	87 (96.7)	90.6–99.3

[a]Abbreviations: 95% CI, 95% confidence interval; HBD, healthy blood donor; C6, C6-ELISA.
[b]Modified BioPlex assay parameters: option 1, FVlsE + p58 at original cutoffs; option 2, FVlsE + p58 at modified cutoffs; option 3, FVlsE only at modified cutoff.
[c]$P < 0.05$, sensitivity/specificity significantly higher than that of Single: Eur-total (data presented in Tables 2 and 3).

composition and cutoffs. To illustrate, an MTTT algorithm with the Biopl/C6 combination would have only a negligible decrease in specificity in HBDs while increasing sensitivity by more than 45% over STTT in EM patients and by more than 10% over an MTTT algorithm with the Euroimmun in the first tier. However, this still necessitates two tests, whereas one would be preferable due to associated costs, practicality, and turnaround time. While our findings are preliminary, they suggest that a modified BioPlex could serve as such with both a sensitivity and a specificity of $>$90%.

A limitation of our study is that we were bound by a case-control design using a convenience sample from a serum bank rather than a consecutive sample collected through a cohort design. Such a one-gate design is preferred for all diagnostic test accuracy studies; however, it is practically impossible to design for LB diagnostics in the absence of a universally accepted reference standard. Similarly, we could not completely circumvent using seropositivity as a criterion for including disseminated LB patients. Even though this may skew toward a higher sensitivity in serological assays, no significant differences in sensitivity were found between well-defined and proven cases. It must also be noted that culture and PCR for *Borrelia* have limited applications and a limited sensitivity, especially for noncutaneous LB manifestations (27). Excluding culture- or PCR-negative cases would therefore have introduced bias as well. It must also be noted that we have not used controls drawn from the general population but rather included HBDs, who are by definition a healthier population subset. We have sought to remedy any potential bias this may have introduced by selecting HBDs from across the Netherlands and from various ages, which resulted in a seropositivity rate comparable to that of previously published background seroprevalences (28). Our cross-reactive/diseased controls, for which only C6 and BioPlex assay data were available, were not from the same area of endemicity as were the cases and HBDs. Instead, these were drawn in the United States as part of the CDC LSR. However, we do not consider this to be a major limitation as their sole reason for inclusion was the presence of specific cross-reactive antibodies, such as rheumatoid factor, antibodies against EBV, or antibodies against *Treponema pallidum*.

As a final thought, it must be noted that the decreased specificity of the BioPlex assays might paradoxically be explained in part by their higher sensitivity. If the BioPlex assays are better at detecting low-level *Borrelia* antibodies in HBDs than their commercial counterparts, then this better detection of the background seroprevalence would show in our data as an increased false-positive rate and therefore lower specificity. For the reasons mentioned above, the limitations of this study design make it challenging to ascertain whether this hypothesis is correct.

In conclusion, our results show that the BioPlex 2200 Lyme IgG and IgM assays are promising tools for the serodiagnosis of EM in Europe. Their combination has an adequate sensitivity in EM patients, even in sera which were collected within 6 weeks after onset of symptoms. However, modifications would be necessary to improve their specificity. Subanalyses suggest that such improvements can be made without a clinically relevant loss in sensitivity. Further research should validate a modified version of the BioPlex assays in a new cohort of European patients and controls.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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