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C6 peptide enzyme immunoassay in Lyme borreliosis serology

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ABSTRACT

The cut-off values used in C6 peptide-based enzyme immunoassay (EIA), a widely used test in Lyme borreliosis (LB) serology, have not been thoroughly analysed. The objective of the study was to examine the performance of the C6 EIA, and to determine optimal cut-off values for the test. The analysed data contained results of 1368 serum samples. C6 EIA index values were compared statistically with the immunoblot (IB) test results. The identified cut-off values were further tested in a well-defined LB patient cohort. Cut-off value 1.6 appeared to be optimal when C6 EIA was used as a stand-alone test. When using C6 EIA as the first-tier test, the optimal cut-off values were 0.9 and 2.4 for negative and positive results. When C6 EIA was used as a second-tier test, samples yielding C6 index values ≥3.0 could be considered positive. The identified cut-off values had also a high sensitivity to identify seropositivity among definite LB patients. The identified cut-off values refine the role of C6 EIA in LB serology. Importantly, the use of C6 EIA leads to a reduction in the number of samples that need to be analysed using an IB, thus also reducing the costs. Two alternative workflows for LB serology including the C6 EIA are suggested.

1. Introduction

Lyme borreliosis (LB) is a tick-borne infection caused by *Borrelia burgdorferi* (*Bb*) *sensu* lato spirochetes (borrelia) (Stanek et al., 2012). Borrelia serology is usually performed using the so-called two-tier testing approach, where the first-tier test is used to identify negative samples from reactive ones, and the second-tier test is used to confirm the results and to identify false positive samples of the first-tier test (Pegalajar-Jurado et al., 2018). First-tier tests are usually based on peptides, recombinant proteins or whole-cell sonicate preparations (WCS) of borrelia, and they are in many cases performed using enzyme immunoassay (EIA) methods. The most commonly used second-tier tests are immunoblots (IB). However, IB testing has several drawbacks, especially the subjectivity of the interpretation of the blots leading to occasional false-positive test results due to over-interpretation of weak bands on the blots. IBs are also more costly than simple EIAs, and they are not performed in all hospital laboratories leading to delays in reporting of the final results.

The so called C6 peptide is a molecule derived from the VlsE surface protein of borrelia. The possibility to use C6 peptide based EIA as the first or the second-tier test in LB serology has been evaluated in several studies (Branda et al., 2011; Lipsett et al., 2016; Branda et al., 2017; Wormser et al., 2013; Wormser et al., 2018; Tjernberg et al., 2007). It has been shown to be a well-performing antigen. However, the interpretation criteria used with the test have not received much consideration, although there is no evidence that the criteria provided by the manufacturer of the most commonly used commercial C6 EIA are optimal, especially when we consider the different requirements for the test when it is used as the first or the second-tier test. Recently, it was demonstrated by Nigrovic and others using pooled data from five studies that there is a correlation between the C6 index value and the diagnosis of LB (Nigrovic et al., 2018). In the analysed studies, C6 EIA was used as the first-tier test. They further showed that higher index values associate with a positive result in the second tier IB analysis. Specifically, C6 index values ≥3.0 were suggested to indicate true LB. The authors were, however, unable to identify a C6 index value with such high specificity

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that there would be no need for a confirmatory second tier IB.

LB is a prevalent infection in Northern Europe. In Finland, the overall incidence of LB was estimated to be 118/100,000 population in 2014 (Sajanti et al., 2017). The highest incidence within the country is reported on the coastal areas of Finland, where the site of the present study (Turku University Hospital, Turku, Finland) is located. In the Clinical Microbiology laboratory of Turku University Hospital, LB serology is based on three tests: An in-house WCS EIA (used since the 1980s) which is performed as the first-tier screening test, C6 EIA (in use since 2012) which is performed as the second-tier test, and a line immunoblot (used in 2008–2017; currently a Luminex based multiantigen assay) which was performed as an additional test when the results of the WCS assay and the C6 EIA were inconclusive (Schulte-Spechtel et al., 2003; Schulte-Spechtel et al., 2004; Schulte-Spechtel et al., 2006). However, the clinicians have the opportunity to request the so-called large serology package, which includes all these tests regardless of the individual test results.

In the present study, the availability of results of the three tests from a large set of samples allowed us to compare the tests with each other. Because our primary objective was to study the performance of C6 EIA in relation to the IB, we used the IB result as the gold standard instead of comparing the C6 index values to clinical diagnoses of the patients. Since IgM antibody reactivity has the tendency to be unspecific, especially after four to six weeks after the infection (Steere et al., 2016; Dessau et al., 2018), and since we did not have detailed information of the infection duration of all patients, IgM results were excluded from the analysis. We evaluated the applicability of C6 EIA as a stand-alone test, as the first-tier test, or as the second-tier test, and determined the optimal cut-off values for the different protocols. Finally, we also tried out the identified cut-off values in a separate, well-defined patient cohort. Based on our results, we suggest two alternative protocols including C6 EIA for LB serology.

2. Methods

2.1. C6 EIA compared to RLB

All consecutive samples sent to our laboratory for LB serology (the large serology package) between 2013 and 2017, and analysed at the time, were included in this retrospective study. Samples that had one or more test results missing were not included to the analysis. The serum samples originated both from general practice and from hospital patients (1368 samples altogether). Data obtained from the samples was handled anonymously and with permission (No T012/006/19) of the hospital district of South-Western Finland. The samples were sent to the laboratory at room temperature and stored at 4 °C until the diagnostic assays were performed. The large serology package includes an in-house WCS IgM and IgG EIA, a commercial EIA based on the C6 peptide (The C6 Lyme ELISA, Oxford Immunotec, Oxford, UK; previously manufactured by Immunetics, Boston, USA), and the recomLine blot (RLB) analysis of IgM and IgG antibodies (Mikrogen GmbH, Neuried, Germany). However, only IgG results were included in this study. Our in-house WCS assay was performed as described previously (van Beek et al., 2018). C6 EIA was performed and interpreted as instructed by manufacturer. C6 EIA resulted in C6 index value which was obtained by dividing the absorbance value of the sample with a standardized factor provided by the manufacturer. RLB was performed as instructed by the manufacturer. Borderline results of RLB were interpreted as positive.

2.2. Evaluation of the identified cut-off values in patient cohort

To evaluate how well the identified cut-off values characterize clinically confirmed LB cases, the identified cut-off values were tested in a patient cohort collected in our previous study (Kortela et al., 2020). The patient cohort consisted of 210 suspected Lyme neuroborreliosis (LNB) patients. In this previous study, 99 of these patients were classified as definite LNB patients with both clinical symptoms of LNB and laboratory confirmation of it (cerebrospinal fluid pleocytosis $\geq$5 leukocytes/mm^3 and intrathecal production of *B. burgdorferi* specific antibodies, or detection of *B. burgdorferi* DNA in the CSF). For the C6 EIA performance evaluation, the tabulated C6 index values were analysed in relation to the clinical diagnosis. The presumption was that the definite acute LNB patients exhibit *Bb* seropositivity allowing us to evaluate especially the sensitivity of the different cut-off values.

2.3. Statistical analysis

The optimal cut-off value for C6 index was determined by comparing the C6 EIA results to the results of RLB, which was used as the gold standard to identify true positives and true negatives. C6 index values were compared both to 1) RLB results of all samples, representing the situation where C6 EIA is used as a stand-alone or as the first-tier test, and 2) RLB results of samples that were positive in the WCS screening assay, representing the situation where C6 EIA is used as the second-tier test. The tests were compared with each other using Receiver operating characteristics (ROC-curve). Statistical analyses were performed with JMP Pro 13 (SAS institute, Cary, NC, USA).

3. Results

Serum samples originated from 726 (53%) women and 642 (47%) men. All age groups were represented among the patients with median age of 56 years old (Q1 = 40.15, Q3 = 68). Age range of the patients was from 0 to 93 years.

3.1. C6 EIA as a stand-alone test or as the first-tier test in two-tier LB serology

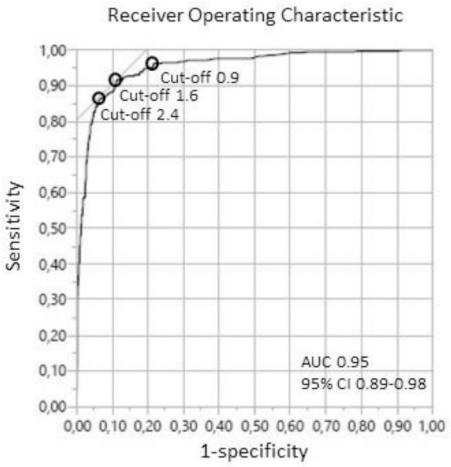
The comparison of the C6 EIA and RLB results of all samples represents the diagnostic set-up where C6 EIA is used as a stand-alone test, or alternatively, as the first-tier test in a two-tier testing protocol. In this comparison, the area under the curve (AUC) of the ROC-curve was 0.95 (95% CI 0.89–0.98) (Fig. 1a). First, we examined the performance of C6 EIA as a stand-alone test. Cut-off value 1.6 resulted in moderately high sensitivity (91%; 95% CI 84–95%) and specificity (89%, 95% CI 81–94%) suggesting that 1.6 would therefore be the optimal cut-off value for C6 EIA as a stand-alone test in LB serology.

Then, we evaluated C6 EIA as the first-tier test in two-tier LB serology. Cut-off index value 0.9 was found to be optimal to identify negative samples due to its high sensitivity (95%; 95% CI 89–98%) (Fig. 1a). The use of this cut-off resulted in a high predictive value of 0.97 (95% CI 0.92–0.99) for IB negativity. On the other hand, the use of cut-off 2.4 resulted in high specificity (94%; 95% CI 88–97%), while the sensitivity (86%; 95% CI 78–91%) remained relatively high. The use of this cut-off value resulted in a predictive value of 0.86 (95% CI 0.78–0.91) for IB positivity. With cut-off values higher than 2.4, the test lost sensitivity dramatically, and therefore no better cut-off value for identifying the positive samples could be found. In summary, the cut-off index value 0.9 can be used to identify negative samples, while the index value $\geq$2.4 identifies true positive samples. Samples yielding index values between 0.9 and 2.4 should be further analysed with a second-tier test.

3.2. C6 EIA as the second-tier test in LB serology

The performance of the C6 EIA as the second-tier test was evaluated using the samples that were positive in the WCS assay (n = 449) with the assumption that samples negative in the WCS assay are directly considered negative without the need to be further analysed. In the ROC curve analysis, the AUC was 0.86 (95% CI 0.78–0.91) (Fig. 2a). The optimal cut-off index value was estimated to be 3.0, which led to sensitivity of 88% (95% CI 80–93%) and specificity of 74% (95% CI

a



Cut-off value	0.9	1.6	2.4
Sensitivity (%) (95% CI)	95 (89-98)	91 (84-95)	86 (78-91)
Specificity (%) (95% CI)	78 (69-85)	89 (81-94)	94 (88-97)
PVIBP (95% CI)	0.67 (0.57-0.75)	0.79 (0.70-0.86)	0.86 (0.78-0.91)
PVIBN (95% CI)	0.97 (0.92-0.99)	0.95 (0.89-0.98)	0.93 (0.86-0.97)
True Positives (n)	412	391	370
False Positives (n)	202	104	59
True Negatives (n)	734	832	877
False Negatives (n)	20	41	62

b

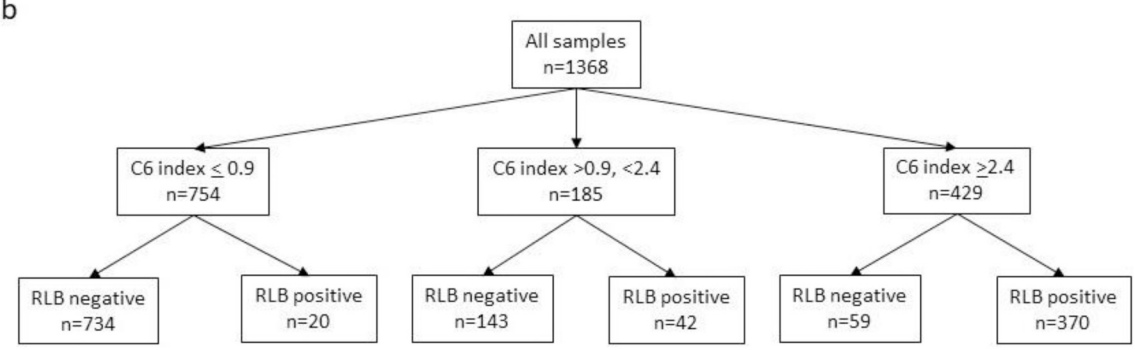


Fig. 1. a) ROC-curve comparing C6 EIA results to RLB results of all 1368 samples. The set-up where C6 EIA is used as a stand-alone or as the first-tier test in LB serology is presented. Cut-off 1.6 resulted in a relatively high sensitivity (91%; 95% CI 84–95%) and specificity (89%, 95% CI 81–94%) and therefore would be optimal when C6 EIA is used as a stand-alone test. Cut-off 0.9 resulted in a high sensitivity (95%; 95% CI 89–98%) and PVIBN (0.97; 95% CI 0.92–0.99), and therefore would perform well as a cut-off value identifying borrelia antibody negative samples. The use of cut-off value 2.4, in turn, resulted in a high specificity (94%; 95% CI 88–97%) and PVIBP (0.86; 95% CI 0.78–0.91), and therefore could be used as a cut-off value for identifying true antibody positive samples. PVIBP = predictive value for immunoblot positivity, PVIBN = predictive value for immunoblot negativity. b) Samples divided into categories according to cut-off values for C6 EIA as the first-tier test in LB serology. Most of the samples with index value ≤0.9 (734/754) would be true negative when RLB is used as the gold standard. Cut-off value 0.9 thus has a high PVIBN. Most of the samples with index value ≥2.4 (370/429) would be true positives. Therefore, cut-off value 3.0 results in a high PVIBP. Of the samples with index value >0.9 and < 2.4, 143/185 would be negative and 42/185 would be positive. Therefore, these samples should be analysed with a second-tier test.

65–82%). With cut-off values ≥3.0, the test started to steeply lose its sensitivity, and with cut-off values <3.0, the specificity decreased rapidly. Importantly, this cut-off resulted in a high predictive value of 0.95 (95% CI 0.89–0.98) for IB positivity, which is in line with the requirements for a second-tier test, while the predictive value for IB negativity was low (0.54, 95% CI 0.44–0.63). Therefore, C6 index value 3.0 seems to be appropriate for the identification of true antibody positive samples when C6 EIA is used as the second-tier test after a WCS EIA as the first-tier test. WCS EIA positive samples with C6 index values <3.0 should be further analysed with a third-tier test.

3.3. *Testing of the identified cut-off values in a well-defined patient cohort*

We also tried out the cut-off values 1.6, 2.4 and 3.0 in a patient cohort collected in our previous study (Kortela et al., 2020). The median C6 index value of the serum samples from the 99 definite LNB patients was 8.3 (Q1 = 6.9, Q3 = 9.0). Index values had range of 1.2 to 10.5. Of the definite LNB patients, 98/99 had C6 index value ≥1.6, 96/99 had index value ≥2.4, and 96/99 showed index value ≥3.0. Therefore, the identified cut-off values had very high sensitivity in this patient cohort (99% (95% CI 95–100%) with cut-off 1.6, and 97% (95% CI 92–99%) with cut-off values 2.4 and 3.0).

4. Discussion

C6 EIA is a well performing and commonly used method in borrelia serology. However, the test could be utilized even more extensively if its sensitivity and specificity could be improved. Both sensitivity and specificity are linked to the interpretation criteria of the test, and by optimizing the cut-off index values used with C6 EIA for different purposes, the performance of the test can be refined. The manufacturer of the most widely used commercial C6 EIA recommends 1.1 as the cut-off index value for identification of *Bb* positive samples, while samples with index values ≤0.9 are to be considered negative. However, the performance of these cut-off values has been evaluated only in one previous publication (Nigrovic et al., 2018).

The results of the present study suggest that the optimal cut-off values for the C6 EIA would be ≤0.9 for negative results and ≥ 2.4 for positive results when the assay is used as the first-tier test in LB serology. As can be seen in Fig. 1b, there were only 59 samples with C6 EIA index values ≥2.4 that were negative in the RLB assay. Thus, index values ≥2.4 can be considered as an indication *Bb* seropositivity with 94% specificity (95% CI 88–97%). In line with this result, Nigrovic *et al* also suggested that cut-off index value 3.0 would be optimal for identifying true LB positive samples (Nigrovic et al., 2018). In this meta-analysis of 4821 patients from 5 different studies, the diagnosis of the patients was

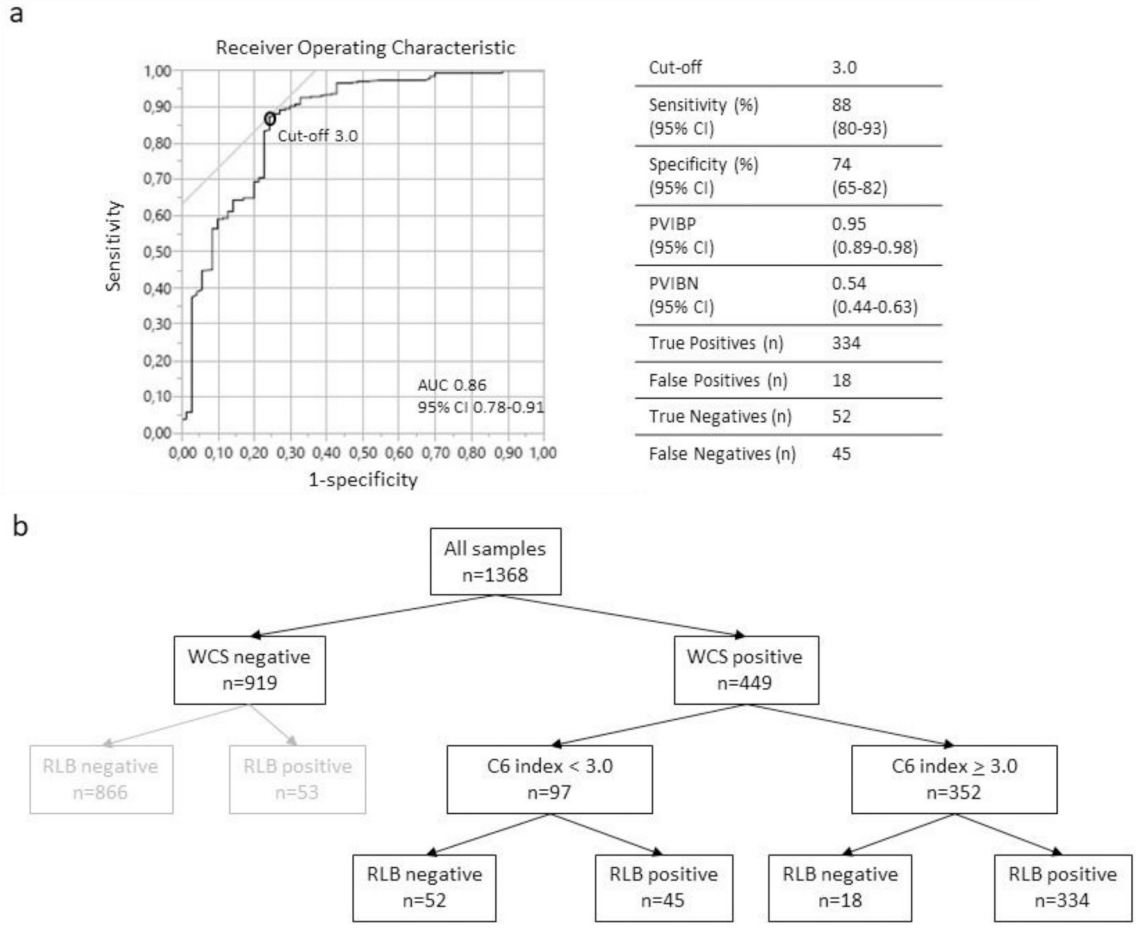


Fig. 2. a) ROC-curve comparing C6 EIA results to RLB results of WCS EIA positive samples, representing the set-up where C6 EIA is used as the second-tier test in LB serology. Cut-off value 3.0 resulted in a high PVIBP (0.95; 95% CI 0.89–0.98), and therefore can be used as a cut-off value for identifying borrelia antibody positive. On the other hand, the cut-off value 3.0 could not correctly categorise negative samples (PVIBN 0.54; 95% CI 0.44–0.63), and the sensitivity (88%; 95% CI 80–93%) and specificity (74%; 95% CI 65–82%) of this cut-off value remained low. PVIBP = predictive value for immunoblot positivity, PVIBN = predictive value for immunoblot negativity. b) Samples divided into categories according to WCS assay result and cut-off value 3.0 when C6 EIA would be used as the second-tier test. In this set-up, after the WCS EIA, 449 samples would be analysed with C6 EIA. Most samples (334/352) with C6 index ≥3.0 would be true positives. Cut-off value 3.0 therefore has a high PVIBP. Of samples with index value <3.0, 52/97 would be true negative and 45/97 would be false negative. These samples should be analysed further with a third-tier test. In addition, most of the WCS assay negative samples (indicated in grey colour) would be true negative samples (866/919), while only a few samples would be falsely classified as negatives (53/919).

compared to C6 index values of their serum samples. While they used the clinical diagnosis of the patient as the gold standard, and our aim was to study whether the IB can be replaced with C6 EIA, both studies resulted in a clearly higher cut-off value than the value suggested by the manufacturer. On the other hand, index values ≤0.9 can be directly interpreted as borrelia antibody negative. The sensitivity of this cut-off is 95% (95% CI 89–98%), and only 20 out of 754 samples with index value ≤0.9 were RLB positive. Samples with C6 EIA index values between 0.9 and 2.4 should, however, be further analysed with, for example, an IB analysis, since 23% (42/185) of the samples with index values between 0.9 and 2.4 were RLB positive, and 77% (143/185) were negative (Fig. 1b). A workflow where C6 EIA is used as the first-tier test is presented in Fig. 3a. An important observation is that using this protocol of C6 EIA as the first-tier test, only 14% (185/1368) of the samples need to be analysed using two different tests.

It has been suggested that C6 EIA could be used as a stand-alone test in LB serology (Lipsett et al., 2016; Wormser et al., 2013). For example, Pegalajar-Jurado *et al* (124 LB patient, including 46 patients with disseminated LB, 347 control samples) found that C6 EIA had 98% sensitivity and 95% specificity for disseminated LB when it was used as a stand-alone test, whereas Branda *et al* (169 LB patients, including 55 patients with disseminated LB, 1300 controls) observed 87% sensitivity and 98% specificity (Pegalajar-Jurado et al., 2018; Branda et al., 2011). Lipsett *et al* (114 paediatric LB patients, 830 control samples) found that C6 EIA had 80% sensitivity and 94% specificity for LB (Lipsett et al., 2016). However, 16 patients (14%) had an early/non-disseminated LB with antibodies possibly only developing at the time of sampling, which may have led to reduced sensitivity. In these studies, where both positive and equivocal results were interpreted as positive and the cut-off value 0.9 was used, strikingly high specificities were reported. In our study, when C6 EIA was used as a stand-alone test, the use of cut-off value 0.9 resulted in an acceptable sensitivity of 95%, while the specificity (78%) was poor. Based on our analysis, the optimal cut-off value for C6 EIA as a stand-alone test would be 1.6 resulting in acceptable sensitivity and specificity. However, with the single cut-off value 1.6, 11% (145/1368) of the samples would be misclassified compared to 6% (79/1368) of the samples when cut-off values 0.9 and 2.4 are used in the two-tier approach. Therefore, C6 EIA might not be optimal as a stand-alone test in LB serology.

Further, our results suggest that the optimal cut-off index value is 3.0 to identify true borrelia antibody positive samples when C6 EIA is used as the second-tier test after a WCS EIA as the first-tier test. The predictive value for IB positivity was high (0.95; 95% CI 0.89–0.98). As can be seen in Fig. 2b, the vast majority of the samples (334/352) testing positive in

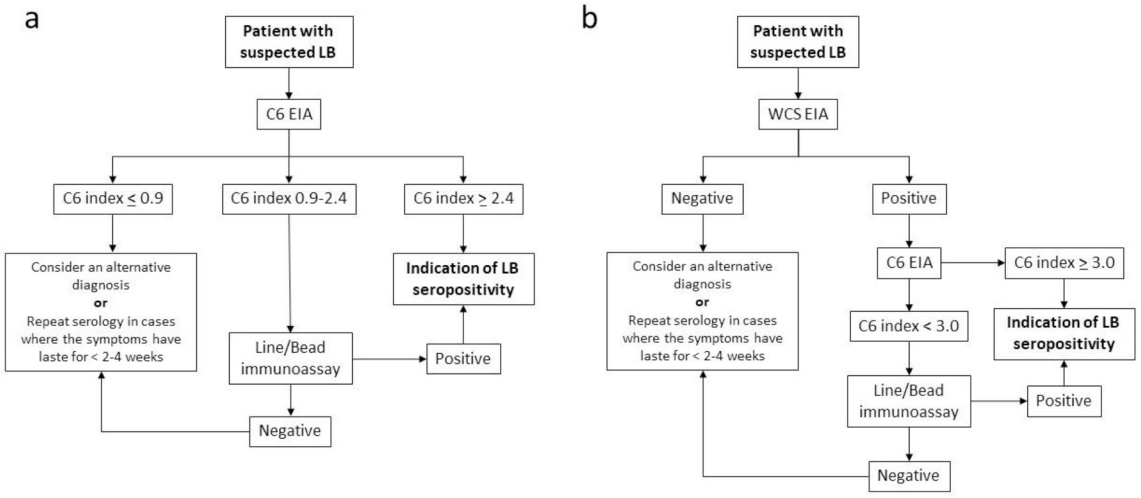


Fig. 3. Proposed workflows for LB serology using C6 EIA. a) When C6 EIA is used as the first-tier test, C6 index values can be divided into three categories: index values ≥2.4 indicate *Bb* seropositivity, index values ≤0.9 indicate negative result, and index values between 0.9 and 3.0 are equivocal results. Samples yielding equivocal results should be further analysed with a second-tier test. b) When C6 EIA is used as a second-tier test, index values ≥3.0 indicate *Bb* seropositivity, while samples with index values <3.0 should be analysed further with a third-tier test (*e.g.* an IB).

C6 EIA with cut-off 3.0 were also positive in RLB analysis. On the other hand, samples with C6 index value <3.0 should be further analysed using a third-tier test, *e.g.* an IB, due to the low predictive value for IB negativity (0.54; 95% CI 0.44–0.63) of the test. Of the samples with C6 EIA result <3.0, 46% (45/97) were positive and 54% (52/97) negative in RLB analysis. A workflow with C6 EIA used as the second-tier test is presented in Fig. 3b. Importantly, however, the protocol including the C6 EIA as the second-tier test after the WCS EIA screening, reduces the number of samples that need to be analysed using an IB from 449 to 97, in other words by almost 80%.

In this study, our primary aim was to investigate whether an IB can be replaced by the C6 EIA. Therefore, we compared the results of different laboratory tests head-to-head instead of comparing the test results against the clinical diagnosis of the patients. However, we tried out the identified cut-off values in a separate well-characterized patient cohort consisting of 210 suspected LNB patients. In this patient cohort, all cut-off values identified definite LNB patients with a high sensitivity. Thus, these cut-off values seem to be highly predictive for clinically diagnosed LNB. One could presume, that these results can be extended to concern also other forms of disseminated LB.

There are some limitations in our study. First, there might be a bias caused by the fact that our data originates from patient samples of which the large serology package including the three tests was requested. The patients may have had more severe or apparent symptoms, which in turn would have prompted the clinicians to request the large serology package. At the same time, the samples included in this study can be considered as a strength. By including samples from both general practice and hospital patients in the analysed material, many different types of patients were represented. Thus, the identified cut-off values can be applied in laboratories that perform LB diagnostics for both primary health care units and hospitals.

Second, the cut-off values that we have determined might not be optimal in other areas of the world. The optimal cut-off values most likely should be determined locally. Our material consisted of samples collected from Finnish patients. In Finland, the proportion of borrelia genospecies (*B. afzelii* as the most abundant and *B. garinii* as the second most abundant genospecies) is very similar to most of the other regions in Europe (Laaksonen et al., 2017). Therefore, the cut-off values we determined could be used in Europe, while different values may need to be defined for samples of US patients. However, Nigrovic and others from the US ended up with a very similar result. This suggests that similar cut-off values might be utilized both in Europe and in North America.

Third, the WCS based EIA used in this study is an in-house assay. However, the WCS assay is based on *B. burgdorferi* s.s. B31-strain, which is used also in commercial assays as the source of the antigen. In addition, we calculated that the sensitivity of our WCS EIA was 88% (95% CI 80–93%) and specificity was 93% (95% CI 86–97%) when RLB was used as the gold standard (379 true positive, 866 true negative, 70 false positive and 53 false negative results). Therefore, our results concerning C6 EIA cut-off values when the test is used as the second-tier test in LB serology are exploitable with any first-tier test with similar performance.

5. Conclusions

In conclusion, when C6 EIA is used as the first-tier test, samples with index values ≤0.9 should be considered negative, and samples with C6 index values ≥2.4 can be interpreted to be borrelia antibody positive (Fig. 3a). Samples with C6 index values between 0.9 and 2.4 should be further analysed with a second-tier test. According to our data, the cut-off 3.0 seems to perform well as a cut-off C6 index value for positivity, when the test is used as the second-tier test. In this set-up, samples yielding index values <3.0 should be further analysed with third-tier test like a line immunoblot, bead immunoassay, or Western blot (Fig. 3b). Importantly, both proposed workflows would significantly reduce the number of samples that would need to be analysed with an IB or a similar, usually more costly, assay. Since C6 EIA is easy to be performed without the requirement of subjective IB signal intensity interpretation, the incorporation of the assays into the LB serology protocol is worth consideration.

Glossary

Enzyme immunoassay (EIA) can be used to detect antibodies in serum samples. An antigen conjugated to enzyme binds to the antibody in the sample and the enzyme converts the added substrate into a detectable substance.

Immunoblot (IB) has pathogen antigens bound on their surface in a specific order. The antibodies in the sample bind to the antigens and can then be detected and identified according to the position of band in the immunoblot.

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Ethics approval

Permission of the hospital district of South-Western Finland (No T012/006/19) was obtained for the study.

Availability of data and material

The datasets generated and analysed during the current study are available in Mendeley Data service.

Author contributions

Meri Rouhiainen: data curation, formal analysis, writing-original draft; Annukka Pietikäinen: data curation, formal analysis, writing-review and editing; Elisa Kortela: data curation, formal analysis; Mari J. Kanerva: data curation, formal analysis; Jarmo Oksi: data curation, formal analysis; Jukka Hytönen: conceptualization, data curation, formal analysis, supervision, writing-review and editing. All authors read and approved the final manuscript.

Declaration of Competing Interest

J.H. is a part time consultant for the diagnostic company Reagena (Toivala, Finland). E.K. reports a non-financial support from MSD, outside the submitted work. The other authors report no conflicts of interest.

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