Laboratory diagnosis of Lyme borreliosis in Scottish patients: a novel approach

S. MAVIN, E. J. WATSON and R. EVANS

National Lyme Borreliosis Testing Laboratory, Raigmore Hospital, Old Perth Road, Inverness IV2 3UJ, UK

Accepted: 24 March 2014

ABSTRACT

Traditional two-tier (enzyme immunoassay [EIA] screening and Western blot confirmation) testing for the laboratory diagnosis of Lyme borreliosis (LB) is expensive, lacks sensitivity in the diagnosis of early LB, cannot distinguish between current and past infection and cannot be used as a marker for treatment response. The aims of the present study is to investigate the role of the C6 EIA as a screening assay, as part of two-tier EIA test strategy, and its use as a marker of treatment response or resolving infection in a routine diagnostic laboratory. The C6 EIA was significantly less sensitive than the Enzygnost Lyme link VlsE/IgG EIA (169/249 *vs*. 190/249 reactive sera, respectively; $P=0.0455$, Fishers exact two-tailed test). The two-EIA strategy, utilising C6 EIA confirmation, was slightly more sensitive than traditional two-tier testing (82/151 *vs*. 67/151 positive sera). Twenty-seven patients were positive by the two-EIA strategy but negative by Western blot, raising questions of specificity, but 12 samples positive with the traditional two-tier testing were negative with the two-EIA strategy. There was no evidence to support the use of the C6 EIA for monitoring treatment response or resolving infection. The authors have devised a novel approach to detect LB in Scottish patients. For cases with a high clinical suspicion of disease, the C6 EIA could be incorporated into a two-EIA strategy, replacing the need for Western blot confirmation with a simpler, more cost-effective two-EIA strategy. Western blot confirmation would be reserved for those patients with discordant EIA results and whose clinical picture is more complex.

KEY WORDS: Borrelia burgdorferi.
Clinical laboratory techniques.
Lyme disease.

Introduction

The National Lyme Borreliosis Testing Laboratory in Inverness, Scotland, uses the internationally recognised two-step testing protocol of a sensitive screening enzyme immunoassay (EIA) and a more specific, confirmatory Western blot for the diagnosis of Lyme borreliosis (LB).[1] However, the traditional two-tier testing strategy is expensive in terms of time and resources, can lack sensitivity in the diagnosis of early LB, is not able to distinguish between current and past infection and therefore cannot be used as a marker for treatment response.[2,3]

VIsE is a variable surface antigen of *Borrelia burgdorferi* thought to be involved in host immune evasion.[4] Gene expression is down-regulated during tick feeding and up-regulated early once the mammalian host has been infected.[4] VIsE, with a predicted molecular mass of approximately 34–35 kDa, contains variable and invariable domains.[5] The sixth invariable region (IR6), which is considered to be conserved among *B. burgdorferi* sensu lato species, contains the C6 peptide, which is highly immunogenic.[6]

The C6 EIA, which utilises a single C6 peptide antigen, has generated a great deal of interest throughout Europe and the USA where it has been proposed as a sensitive screening assay,[7,8] as an alternative to traditional two-tier testing as part of a two-tier EIA test strategy[9–11] and an indirect marker of treatment response/resolving infection.[12]

The aims of this study are to investigate the role of the C6 assay as a screening assay and as part of a two-tier EIA test strategy and its use as a marker of treatment response or resolving infection in a routine diagnostic laboratory.

Materials and methods

To determine C6 EIA sensitivity, sera ($n=249$) from patients with clinically suspected Lyme borreliosis, referred to the National Lyme Borreliosis Testing Laboratory, Raigmore Hospital, Inverness, from laboratories throughout Scotland during 2012 and 2013 were tested both by the C6 Lyme EIA (Immunetics, Boston MA, USA) and the current screening test, the Enzygnost Lyme link VlsE/IgG (a whole-cell EIA with additional VlsE antigens; Siemens Healthcare Diagnostics Products, Marburg, Germany), as per the manufacturers' instructions. The C6 Lyme EIA was tested manually whereas the Enzygnost Lyme link VlsE/IgG was tested on the Dynex DS2 platform (Launch Diagnostics, Kent, UK). The results were compared using the two-tailed Fishers exact test.

To assess the potential of a two-EIA testing strategy (whole-cell EIA followed by C6 Lyme EIA confirmation), the results from 151 of the above sera that were tested by the traditional two-tier testing strategy utilising Enzygnost Lyme link VlsE/IgG screening with manual commercial EU Lyme IgG Western blot (Trinity Biotech, Co Wicklow, Ireland) confirmation, were compared with the results that would have been obtained with the two-EIA strategy. Statistical analysis was carried out using the two-tailed Fishers exact test.

Correspondence to: Ms. Sally Mavin
Email: sally.mavin@nhs.net

To assess the use of the C6 EIA as an indirect marker of treatment response/resolving infection, 76 archived sera from 23 patients (2–6 sera per patient, taken three months to seven years apart) were tested with the C6 Lyme EIA and the results were compared with traditional two-tier testing of Enzygnost Lyme Link VlsE/IgG EIA followed by in-house IgG Western blot confirmation (whole-cell lysate blot incorporating local *B. burgdorferi* sensu stricto and *B. afzelii* antigen, 50:50 mixture).[13]

Results

C6 as a screening assay

Overall, the C6 EIA was significantly less sensitive than the Enzygnost Lyme link VlsE/IgG EIA (169/249 *vs.* 190/249 reactive sera, respectively, $P=0.0455$).

Two-EIA strategy

The two-EIA strategy was more sensitive (although not significantly) than the traditional two-tier strategy, detecting 82/151 *vs.* 67/151 positive sera, respectively ($P=0.1069$; Table 1). Of the 27 sera positive by the two-EIA strategy but not by the traditional two-tier testing (Table 1), 17 were from patients with symptoms of early LB (nine erythema migrans [EM], seven rash, one meningitis), two with late LB (arthritis/joint pain), five of past infection/non-specific symptoms, and three with insufficient clinical information. Conversely, 12 sera were positive with the traditional two-tier testing but not with the two-EIA strategy (Table 1). Four were from patients with symptoms of early LB (two EM, one rash, one neuroborreliosis), two with late LB (arthritis/joint pain), five of past infection, and one with insufficient clinical information.

C6 as a marker of treatment response or resolving infection

Of the 23 patients with multiple archived sera taken at different time points that were tested by traditional two-tier testing (Enzygnost Lyme link VlsE/IgG EIA followed by in-house IgG Western blot) then C6 Lyme EIA, nine patients had no change in Enzygnost or C6 EIA titre or Western blot band intensity over time. There was a decrease in EIA titre and/or Western blot band intensity in all other patients (Table 2), although this was only qualitative (i.e., positive to negative) in four patients (three had a change in WB result, one had a change both in Enzygnost and C6 EIA results).

Discussion

The C6 EIA has been found to be sensitive in studies in the USA,[11,12] but in Europe the C6 EIA is said to be less sensitive than other more traditional whole-cell EIAs as not all patients have a response to the C6 peptide.[14–16] Although the IR6 region is conserved, there appears to be variation in 4–5 amino acid sequences among genospecies.[17] This would be less of a problem for the USA where *B. burgdorferi* sensu stricto is the sole pathogenic species, but would explain the differences in Europe where there are at least three pathogenic species.[14–16,18] The results presented here indicate that in Scottish patients the C6 EIA is less sensitive than the Enzygnost Lyme link VlsE/IgG screening EIA, and at the time of this study it is almost four times more expensive.

Table 1. Results of two-EIA (Enzygnost Lyme link VlsE/IgG EIA and C6 Lyme EIA confirmation) versus traditional two-tier testing (Enzygnost Lyme link VlsE/IgG EIA and Trinity IgG Western blot confirmation).

		Traditional two-tier testing			
		Pos	Ind	Neg	Total
Two-EIA testing	Pos	55	20	7	82
	Equiv	3	1	0	4
	Neg	9	6	50	65
	Total	67	27	57	151

In the USA it has also been suggested that the C6 EIA could be a standalone test,[11,19,20] without the need for Western blot confirmation, as it is at least as sensitive as traditional two-tier testing in early LB.[3,6,11,21] An immune response to the C6 peptide appears at an early point in time, often developing more rapidly than either the IgM or IgG response to a combination of antigens on which the two-tier blot criteria are based.[20] However, like other EIAs, Western blot is still more specific than the C6 EIA.[11,22] As the C6 EIA is less specific than traditional two-tier testing,[10,20] this approach could produce more false-positive results and contribute further to the over diagnosis of Lyme borreliosis.

To reduce this false-positive rate, a two-EIA approach (whole cell EIA with C6 EIA confirmation) has been proposed,[9,10,23] which was claimed to provide sensitivity close to that of the C6 EIA alone in early LB, but maintain the specificity of traditional two-tier testing. Indeed, the two-EIA approach performed better in early LB (i.e., it was more sensitive) than traditional two-tier testing and was comparable in late LB.[10] The two-EIA approach would be very desirable for the routine laboratory as it is simpler and more objective than traditional two-tier testing, reducing the need for experienced laboratory staff and equipment. Instead of referring samples to a reference laboratory for screening and/or Western blot confirmation, the EIA approach would enable the routine laboratory to do its own testing and confirmation, reducing costs and turnaround times. The present results indicate that traditional two-tier testing is slightly less sensitive than the two-EIA strategy, although this may be due in part to poor sensitivity of the commercial Western blot assay used.

Encouragingly, the clinical information for those sera positive with the two-EIA strategy only suggested that the majority of patients (70%) had LB. However, it is of concern that 12 samples positive with the traditional two-tier testing were missed with the two-EIA strategy as they were C6 EIA negative, six (50%) with symptoms of current LB.

Table 2. Assays with decreased titre/weaker bands following treatment.

Assay	No. patients
Enzygnost and C6 EIA, Western blot	9
C6 and Enzygnost EIA	1
C6 EIA and Western blot	1
C6 EIA only	1
Enzygnost EIA only	2

Unfortunately, specificity could not be determined in this study as the study sera were from routine samples that could not easily be defined.

These results would suggest that while there may be a place for the two-EIA strategy within the routine laboratory due to the increasing pressures of reduced staff and resources, there is still a need for Western blot in certain situations. The authors have devised an algorithm (Fig. 1) in which the two-EIA strategy could be employed for patients with a high clinical suspicion of LB (i.e., with tick bite and rash). Although serological testing is not advocated for patients with the erythema migrans rash diagnostic of Lyme borreliosis, the reality in Scotland is that many general practitioners are not familiar with this characteristic rash, or its many atypical forms, and still require laboratory confirmation. The testing algorithm reserves Western blot testing for those patients with discordant EIA results and those whose clinical picture is not straightforward, such as those with non-specific symptoms or those with late LB. As well as being very specific, the Western blot provides additional valuable information such as the degree of expansion of the antibody response.[10,22]

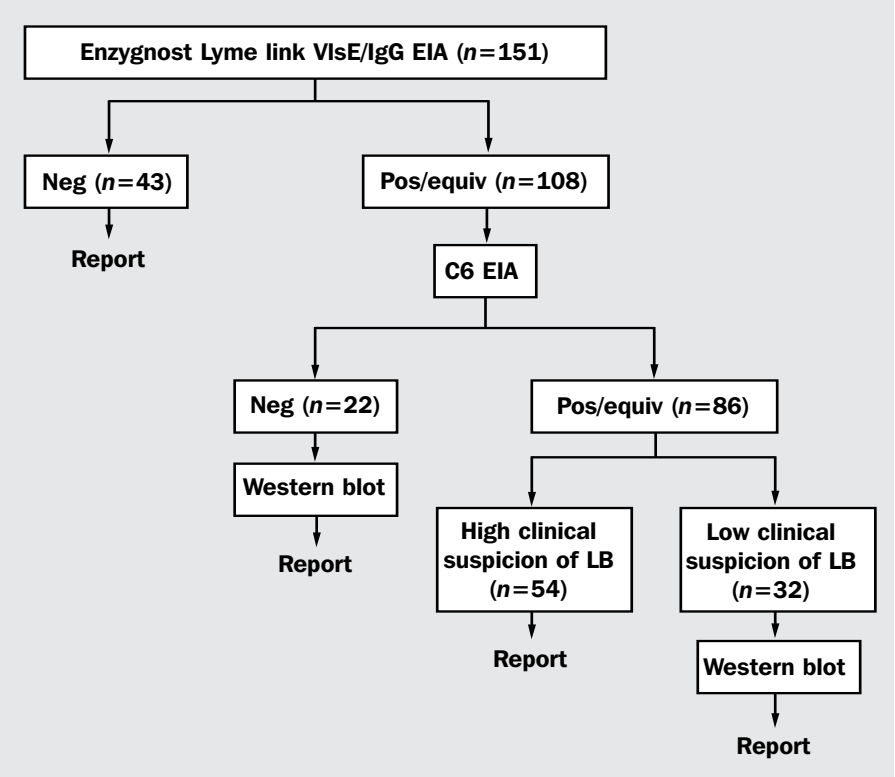

Fig. 1. Proposed Lyme borreliosis testing algorithm.

The algorithm is, in part, similar to that suggested by Jansson *et al.*, who suggested a two-EIA approach using WB confirmation for discordant samples only.[9] If the proposed algorithm had been applied to the 151 sera originally tested with the traditional two-tier strategy, it would have reduced the number of sera requiring Western blot by 50% (108 to 54), saving approximately £600 in reagent costs, with additional saving on staff time. Perhaps more importantly, it would allow routine laboratories to do their own testing and confirmation for the majority of their LB patients, reducing turnaround times and the testing burden for reference laboratories, ensuring there can be more focus on the more complex cases.

Traditional serological detection of *B. burgdorferi* antibodies is not recommended for monitoring treatment response or resolving infection because IgG antibodies can remain elevated for years. However, small differences can occasionally be observed when testing sequential samples in parallel following treatment. A more robust method for monitoring treatment response or resolving infection would be extremely beneficial for patient management, especially in complexes cases of late LB; therefore, the proposal that the C6 EIA may be used in this capacity was encouraging.

When the C6 EIA results from 23 patients with multiple sera were examined there was no evidence to support that the C6 EIA provides any information above that provided by the traditional two-tier testing for monitoring treatment response or resolving infection. This was similar to a study of Lyme arthritis in children in which the rate of decline of antibodies to IR6 did not appear useful in the prediction of the treatment response or the clinical course of Lyme arthritis,[24] and indeed the majority of patients were still positive for IR6 four years after diagnosis.

Unfortunately, the studies that implicated the C6 EIA in this role were performed on serial dilutions of sera, and were not based on C6 antibody index comparisons, which would make it much more difficult and complex for the routine laboratory.[2,12,25,26] Philipp *et al*. originally stated that there was a decline in C6 antibody titres by greater than four-fold in all successfully treated LB study patients at 20 weeks or more.[12] However, it was later conceded that as infection progresses the quantitative C6 test used to assess response to treatment decreased in sensitivity so that a decline in C6 antibody titre occurred only in successfully treated patients with early localised or early disseminated LB,[25] and not in patients with late LB, where such information would clinically be of more use.

Although these findings were promising, Kannian *et al*. found that the rate of decline of C6 antibodies was usually too slow to be useful at the time that decisions about further antibiotic therapy needed to be made in patients with Lyme arthritis.[27] The researchers also highlighted that a great proportion of patients will have a persistently positive C6 test and that this should not be equated with persistence of infection.[27] As a result, a single titre is not informative of status after therapy and it can be used only as part of a longitudinal assessment of a patient.[2] It is important to note that the C6 EIA varied between the studies examined; some were in-house whereas others were commercial, and they varied slightly in the IR6 sequences used, which could affect assay sensitivity and specificity.

In conclusion, although the C6 EIA appears to be sensitive, there is no convincing evidence to suggest that it detects infection earlier than other assays, infection may be missed

in some European patients due to potential IR6 variation, and it may lack some specificity. The C6 EIA may be used to monitor treatment response but a large decline in C6 after treatment appears to be less common in late LB, where this may be of most use.

The present study introduces a novel approach to detect Lyme borreliosis in Scottish patients. Although Western blot remains invaluable for the more complex cases of Lyme borreliosis, the authors have shown that for straightforward cases of Lyme borreliosis in Scotland, the C6 EIA could play an important role in the routine laboratory, replacing the need for Western blot confirmation undertaken by a reference laboratory with a simpler, more cost-effective two-EIA strategy. □

The authors would like to thank all clinicians and laboratory staff within Scotland who continue to support the laboratory.

References

1 Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. *MMWR. Morbidity and Mortality Weekly Report* 1995; **44** (31): 590–1.

2 Philipp MT, Marques AR, Fawcett PT, Dally LG, Martin DS. C 6 test as an indicator of therapy outcome for patients with localized or disseminated Lyme borreliosis. *J Clin Microbiol* 2003; **41** (11): 4955–60.

3 Bacon RM, Biggerstaff BJ, Schriefer ME *et al*. Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. *J Infect Dis* 2003; **187** (8): 1187–99.

4 Singh SK, Girschick HJ. Molecular survival strategies of the Lyme disease spirochete *Borrelia burgdorferi*. *Lancet Infect Dis* 2004; **4** (9): 575–83.

5 Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of Lyme borreliosis. *Clin Microbiol Rev* 2005; **18** (3): 484–509.

6 Ting Liang F, Steere AC, Marques AR, Johnson BJB, Miller JN, Philipp MT. Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* VlsE. *J Clin Microbiol* 1999; **37** (12): 3990–6.

7 Smismans A, Goossens VJ, Nulens E, Bruggeman CA. Comparison of five different immunoassays for the detection of *Borrelia burgdorferi* IgM and IgG antibodies. *Clin Microbiol Infect* 2006; **12** (7): 648–55.

8 Tjernberg I, Krüger G, Eliasson I. C6 peptide ELISA test in the serodiagnosis of Lyme borreliosis in Sweden. *Eur J Clin Microbiol Infect Dis* 2007; **26** (1): 37–42.

9 Jansson C, Carlsson S, Granlund H, Wahlberg P, Nyman D. Analysis of *Borrelia burgdorferi* IgG antibodies with a combination of IgG ELISA and VlsE C6 peptide ELISA. *Clin Microbiol Infect* 2005; **11** (2): 147–50.

10 Branda JA, Linskey K, Kim YA, Steere AC, Ferraro MJ. Two-tiered antibody testing for Lyme disease with use of 2 enzyme immunoassays, a whole-cell sonicate enzyme immunoassay followed by a vlse c6 peptide enzyme immunoassay. *Clin Infect Dis* 2011; **53** (6): 541–7.

11 Mogilyansky E, Loa CC, Adelson ME, Mordechai E, Tilton RC. Comparison of Western immunoblotting and the C6 Lyme antibody test for laboratory detection of Lyme disease. *Clin Diagn Lab Immunol* 2004; **11** (5): 924–9.

12 Philipp MT, Bowers LC, Fawcett PT *et al*. Antibody response to IR6, a conserved immunodominant region of the VlsE lipoprotein, wanes rapidly after antibiotic treatment of *Borrelia burgdorferi* infection in experimental animals and in humans. *J Infect Dis* 2001; **184** (7): 870–8.

13 Mavin S, McDonagh S, Evans R, Milner RM, Chatterton JM, Ho-Yen DO. Interpretation criteria in Western blot diagnosis of Lyme borreliosis. *Br J Biomed Sci* 2011; **68** (1): 5–10.

14 Marangoni A, Moroni A, Accardo S, Cevenini R. *Borrelia burgdorferi* VlsE antigen for the serological diagnosis of Lyme borreliosis. *Eur J Clin Microbiol Infect Dis* 2008; **27** (5): 349–54.

15 Tjernberg I, Sillanpää H, Seppälä I, Eliasson I, Forsberg P, Lahdenne P. Antibody responses to Borrelia IR6 peptide variants and the C6 peptide in Swedish patients with erythema migrans. *Int J Med Microbiol* 2009; **299** (6): 439–46.

16 Sillanpää H, Lahdenne P, Sarvas H *et al*. Immune responses to borrelial VlsE IR6 peptide variants. *Int J Med Microbiol* 2007; **297** (1): 45–52.

17 Göttner G, Schulte-Spechtel U, Wilske B. Heterogeneity of the immunodominant surface protein VlsE among the three genospecies of Borrelia burgdorferi pathogenic for humans. *Int J Med Microbiol* 2004; **293** (Suppl 37): 172–3.

18 Wormser GP, Liveris D, Hanincová K *et al*. Effect of Borrelia burgdorferi genotype on the sensitivity of C6 and 2-tier testing in North American patients with culture-confirmed Lyme disease. *Clin Infect Dis* 2008; **47** (7): 910–4.

19 Branda JA, Aguero-Rosenfeld ME, Ferraro MJ, Johnson BJ, Wormser GP, Steere AC. 2-tiered antibody testing for early and late Lyme disease using only an immunoglobulin G blot with the addition of a VlsE band as the second-tier test. *Clin Infect Dis* 2010; **50** (1): 20–6.

20 Wormser GP, Schriefer M, Aguero-Rosenfeld ME *et al*. Single-tier testing with the C6 peptide ELISA kit compared with two-tier testing for Lyme disease. *Diagn Microbiol Infect Dis* 2013; **75** (1): 9–15.

21 Lawrenz MB, Hardham JM, Owens RT *et al*. Human antibody responses to VlsE antigenic variation protein of *Borrelia burgdorferi*. *J Clin Microbiol* 1999; **37** (12): 3997–4004.

22 Steere AC, McHugh G, Damle N, Sikand VK. Prospective study of serologic tests for Lyme disease. *Clin Infect Dis* 2008; **47** (2): 188–95.

23 Nyman D, Willén L, Jansson C, Carlsson SA, Granlund H, Wahlberg P. VlsE C6 peptide and IgG ELISA antibody analysis for clinical diagnosis of Lyme borreliosis in an endemic area. *Clin Microbiol Infect* 2006; **12** (5): 496–7.

24 Heikkilä T, Huppertz H, Seppälä I, Sillanpää H, Saxen H, Lahdenne P. Recombinant or peptide antigens in the serology of Lyme arthritis in children. *J Infect Dis* 2003; **187** (12): 1888–94.

25 Philipp MT, Wormser GP, Marques AR *et al.* A decline in C6 antibody titer occurs in successfully treated patients with culture-confirmed early localized or early disseminated Lyme borreliosis. *Clin Diagn Lab Immunol* 2005; **12** (9): 1069–74.

26 Peltomaa M, McHugh G, Steere AC. Persistence of the antibody response to the VlsE sixth invariant region (IR6) peptide of *Borrelia burgdorferi* after successful antibiotic treatment of Lyme disease. *J Infect Dis* 2003; **187** (8): 1178–86.

27 Kannian P, McHugh G, Johnson BJB, Bacon RM, Glickstein LJ, Steere AC. Antibody responses to *Borrelia burgdorferi* in patients with antibiotic-refractory, antibiotic-responsive, or non-antibiotic-treated Lyme arthritis. *Arthritis Rheum* 2007; **56** (12): 4216–25.