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The development of an IgG avidity Western blot with potential to differentiate patients with active Lyme borreliosis from those with past infection

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Title: The development of an IgG avidity Western blot with potential to differentiate patients with active Lyme borreliosis from those with past infection.

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Abstract

Objectives: Current serological methods cannot distinguish active from past infection with *Borrelia burgdorferi* sensu lato. The aim of this study was to develop an IgG avidity Western blot and assess its potential to differentiate patients with early and late Lyme borreliosis (LB) i.e. active disease, from those infected in the past.

Methods: An IgG avidity Western blot was developed. Penalized linear discriminant analysis (PLDA) was employed to compare the Western blot/avidity Western blot profiles of an evaluation panel consisting of 75 sera from patients with early (n=26) and late (n=24) LB and past infection (n=25). The PLDA models produced were used to predict infection stage for 20 well characterised sera from the Centers for Disease Control and Prevention (CDC) Lyme disease serum repository and 112 routine seropositive sera (disease stage unknown), to validate and assess the usefulness of the avidity Western blot/avidity Western blot and PLDA approach.

Results: PLDA correctly classified 40/51 (78%) of patients when early LB and past infection groups in the evaluation panel were compared. Likewise, when late LB and past infection groups were compared, 34/49 (69%) were correct. The resultant PLDA models correctly predicted infection stage for 18/20 (90%) of the CDC sera, validating the use of the avidity Western blot/avidity Western blot and PLDA approach. When tested with the routine sera, 21/29 (72%) tested with the early LB vs. past infection model were correct but only 32/83 (39%) with the late LB vs. past infection model. Past infection was predicted for 40/112 (35%) of the routine sera, 80% of which correlated with the clinical picture.

Conclusion: The Western blot/ avidity Western blot with PLDA approach shows exciting potential for being able to predict disease stage in some patients with LB, which could improve patient management.

Keywords: *Borrelia burgdorferi*, IgG avidity, Laboratory diagnosis, Lyme borreliosis,

Western blot

ACCEPTED MANUSCRIPT

Introduction

The management of patients suspected of having Lyme borreliosis (LB) can pose a challenge to the clinician. While the presence of the typical erythema migrans rash following a tick bite is clinically diagnostic of LB (Stanek et al., 2011), serology to detect the presence of antibodies to *Borrelia burgdorferi* sensu lato (s.l.) is used to support a clinical diagnosis of LB in most other cases. Testing follows an internationally accepted two-step approach (Centers for Disease Control and Prevention, 1995): a sensitive screening assay followed by a confirmatory immunoblot (Moore et al., 2016). Both IgM and IgG immunoblots can be used for confirmation, however, IgM blots tend to have poorer specificity and a prolonged elevation of IgM antibodies can occur despite effective antimicrobial treatment (Kalish et al., 2001, Shapiro and Gerber, 2000). The concentration of IgG antibodies may decline after treatment, but even after the patient is clinically cured these antibodies often remain detectable for many years (Kalish et al., 2001, Shapiro and Gerber, 2000). As a result, serological testing cannot readily distinguish active from past and treated infection or from re-infection occurring after a prior treated infection and hence provide a definitive diagnosis (Moore et al., 2016). This means that a positive result in those patients with persisting non-specific symptoms who do not fit within the strict case definitions for LB (Stanek et al., 2011) is difficult for the laboratory and clinician to interpret and is of limited value for the management of these patients. Consequently, the identification of markers of current infection has been highlighted as an important area of LB research (Moore et al., 2016, Poggensee et al., 2008).

Avidity testing is used to determine the stage of infection for a range of organisms, including *Toxoplasma gondii* and cytomegalovirus. During early infection the antibodies that are produced bind with low avidity. As the infection progresses, antibodies with higher avidity

are selected for and predominate. Although ELISA avidity assays have been used experimentally to differentiate between early and late LB (Basse Guérineau, Dhôte et al. 1999, Luyasu, Mullier et al. 2001), avidity immunoblot assays, which have the advantage in that they can measure antibody avidity to a range of individual antigens, have not been similarly utilised. The aim of this study was to develop an IgG avidity Western blot and assess its potential to differentiate patients with early and late LB (i.e. active LB) from those infected in the past but who are symptomatic.

Methods

Samples

Sera included in the study had been referred to the National Lyme Borreliosis Testing Laboratory (NLBTL), Raigmore Hospital, Inverness, UK from laboratories and clinicians throughout Scotland for routine testing. Ethical approval was not required. Sera had been screened for the presence of *B. burgdorferi* s.l. antibodies by commercial EIA, either ZEUS ELISA *Borrelia burgdorferi* IgG/IgM test system (Zeus Scientific, Somerville, New Jersey, USA) or Enzygnost Lyme link VlsE/IgG ELISA (Siemens, Surrey, UK). EIA reactive sera were confirmed by an in-house IgG Western blot assay, which uses a 50:50 antigen mix of local *B. burgdorferi* sensu stricto (s.s.) and *B. afzelii* (Mavin et al., 2009). The Western blot results were interpreted according to current published guidelines (Mavin et al., 2011). Only Western blot positive sera (seropositive sera) were included in the study.

Avidity Western blot development

Two blotting test strips were set up in parallel for each serum tested. The strips were prepared using a 50:50 antigen mix of local *B. burgdorferi* s.s. and *B. afzelii*, as previously described

(Mavin et al., 2011). The first strip was tested by the standard in-house IgG Western blot protocol (Mavin et al., 2009, Davidson et al., 1996). For the second (avidity) strip, the Western blot protocol was adapted by substituting phosphate buffered saline-Tween [PBST, 1xPBS with 0.05% (v/v) Tween 20 (Sigma Aldrich, Poole, UK)] with an avidity reagent (urea/PBST) for the first wash step after overnight incubation of test strips with test serum to remove any antibodies bound with low avidity to the proteins on the blot strip. After the test was complete, the blot strips were scanned (GS800 scanning densitometer, Bio-Rad, Hemel Hempstead, UK), and analysed with Quantity One software (Bio-Rad). Only those bands representing serum IgG antibodies bound to borrelia-specific proteins on the blot strips, measured to have a relative density of >0.117 , were recorded. The band intensities on the avidity strip were compared with those on the standard Western blot strip for each serum.

Different aspects of the assay were investigated, including: urea concentration (4 M, 6 M and 8 M, Sigma); membrane type (nitrocellulose versus polyvinyl-difluoride, PVDF, 0.45 μ M, GE Healthcare Whatman, Maidstone, UK) and sample dilution (1:50, 1:100 and 1:150).

Avidity parameters (i.e. what represents a low or a high antibody response) were also assessed.

Avidity Western blot evaluation

A panel of 75 sera from: 26 patients assigned as early LB; 24 late LB and 25 past infection were used to evaluate the avidity Western blot assay. Patients were assigned to the three patient groups by two of the authors (RE and SM) based on all available clinical details and information from any accompanying questionnaires, correspondence and samples. Patients were assigned as early LB if the patient had an accompanying questionnaire where the clinician had determined that the patient had LB, and where the clinical symptoms were for

less than 6 months: 22 patients had erythema migrans, 1 patient neuroborreliosis and 3 flu-like illness following a tick bite. The majority of these patients (17/26, 65%) were sampled 2-8 weeks post tick bite/ onset of symptoms, one patient was sampled within 2 weeks and 7/26 (27%) after 2-5 months. There was no date of onset for one patient with EM.

Likewise, patients were assigned as late LB if the patient had an accompanying questionnaire where the clinician had determined that the patient had LB, and where the clinical symptoms were present for more than 6 months (as per EFNS classification)(Mygland et al., 2010): 3 patients had acrodermatitis chronic atrophicans, 1 patient had borrelia lymphocytoma, 2 had long-standing EM and the remaining 18 patients had joint and/or neurological symptoms. Patients were categorised as having evidence of past infection if the clinician had determined that the patient did not currently have LB, although seropositive.

The Western blot and avidity Western blot data from the sera in the evaluation panel was documented in IBM SPSS (Statistical Package for the Social Sciences). These included: the presence or absence of an antibody reaction to individual proteins in each Western blot strip (total IgG antibodies) in addition to the presence of low or high avidity antibodies to each of these proteins, as determined by the avidity strip. Only antibody responses to those proteins considered to be specific for *B. burgdorferi* s.l. were included (92, 58, 48, 46, 43, 41, 39, 34, 32, 30, 26, 22, 20 and 18kDa)(Mavin et al., 2011). The proportion of samples that detected each *B. burgdorferi* s.l. protein by antibodies in the Western blot and with high and low avidity antibodies was then compared for the three patient groups (early LB, late LB and past infection), using the Mann-Whitney U Test to identify which of these were significantly different between the groups.

Discriminant analysis was employed to compare the Western blot and avidity Western blot results from the sera for the 3 patient groups in the evaluation panel to statistically predict whether a patient had early or late LB or past infection and to identify any important predictor variables. Because of the small number of data and the large number of predictor variables, we used a penalized version of linear discriminant analysis (PLDA) to limit the risk of over-fitting the data. Three different analyses were performed. Firstly, the banding profiles from all 3 patient groups were compared in one analysis. Then, the banding profiles from the early and late LB groups were compared with each other and then with those from the past infection group in turn. PLDA was conducted in R version 3.1.0 GUI for Mac OS X 1.64 Snow Leopard (2014-04-10) using library “penalizedLDA” version 1.0 and library “flsa” version 1.05 from CRAN. The 48 predictor variables included in the analyses were:

- (i) 45 covariates expressing the detection of 15 borrelia-specific proteins as per Mavin *et al* (2011) [8](92, 58, 48, 46, 43, 41, 39, 34, 32, 30, 28, 26, 22, 20 and 18kDa respectively) by antibodies in the Western blot, high avidity antibodies and low avidity antibodies, coded as 0 (undetected) or 1 (detected)
- (ii) the number of proteins detected per sample by antibodies in the Western blot
- (iii) the number of proteins detected per sample with high avidity antibodies
- (iv) the number of proteins detected per sample with low avidity antibodies

The resulting PLDA predictive models were assessed using the Leave-one-out cross validation approach.

Western blot/ avidity Western blot and PLDA validation and testing

The PLDA predictive models that had been trained on the evaluation panel data were tested on two additional data sets to predict the disease status from the Western blot and avidity Western blot results. Firstly, to further validate the Western blot/avidity Western blot and

PLDA approach, the model predictions were tested on a high-confidence data set of an additional 20 IgG immunoblot positive sera, obtained from the Centers for Disease Control and Prevention (CDC) Lyme disease serum repository (Fort Collins, Colorado, USA), which is available for researchers for the validation of novel diagnostic tests for LB (Molins et al., 2014): 12 from patients with confirmed early LB (8 erythema migrans, 2 early neurological, 2 cardiac) and 8 with late LB (arthritis).

Secondly, to assess the usefulness of the Western blot/avidity Western blot and PLDA approach in the routine diagnostic setting and to observe how the avidity Western blot profile may change over time in individual patients, the model predictions were tested on a lower-confidence data set, which consisted of a further 112 routine clinical samples obtained from NLBTL, seropositive for *B. burgdorferi* s.l. with stage of infection unknown: 36 single serum samples from patients with clinically suspected LB from January to May 2012; 76 stored sera from 23 patients (each patient had 2 or more seropositive sera, taken months to years apart). The model predictions were then compared with the clinical information provided for each patient.

Results

Avidity Western blot development

A graded effect was seen with the avidity reagent as the number of proteins detected in each sample by avidity Western blot decreased as the urea concentration increased from 4 M to 6 M and 8 M. The 8 M urea produced the largest effect (i.e. removed the most amount of antibodies) so was chosen for use in the study. Nitrocellulose membrane was chosen because it produced cleaner results than PVDF, was much cheaper and it ensured that the Western

blot and avidity Western blot procedures remain as similar as possible. Likewise, sample dilutions for the study were kept at 1:50 (as for the Western blot) as little difference was observed between the 1:50, 1:100 and 1:150 dilutions, again minimising differences between the two assays to allow a true comparison. Following extensive investigations of different avidity parameters, a decrease in intensity for any individual band of more than 75% was considered to represent a low avidity antibody response to that particular borrelia protein. A band that decreased in intensity less than 75% was considered to be a high avidity antibody response. Figure 1 depicts the optimised Western blot/ avidity Western blot protocol.

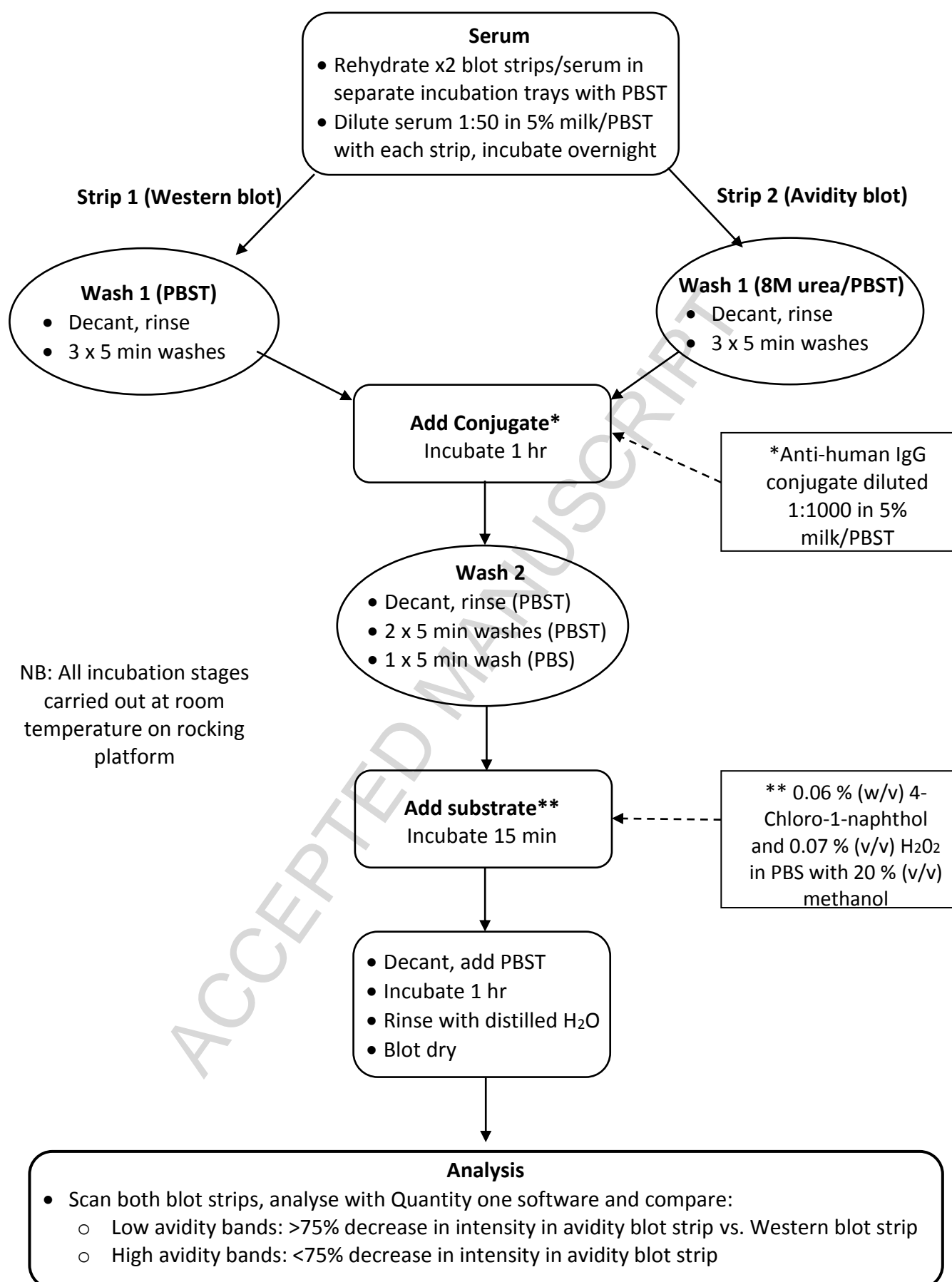


Figure 1: Flow-chart depicting the optimised Western blot/ avidity Western blot protocol

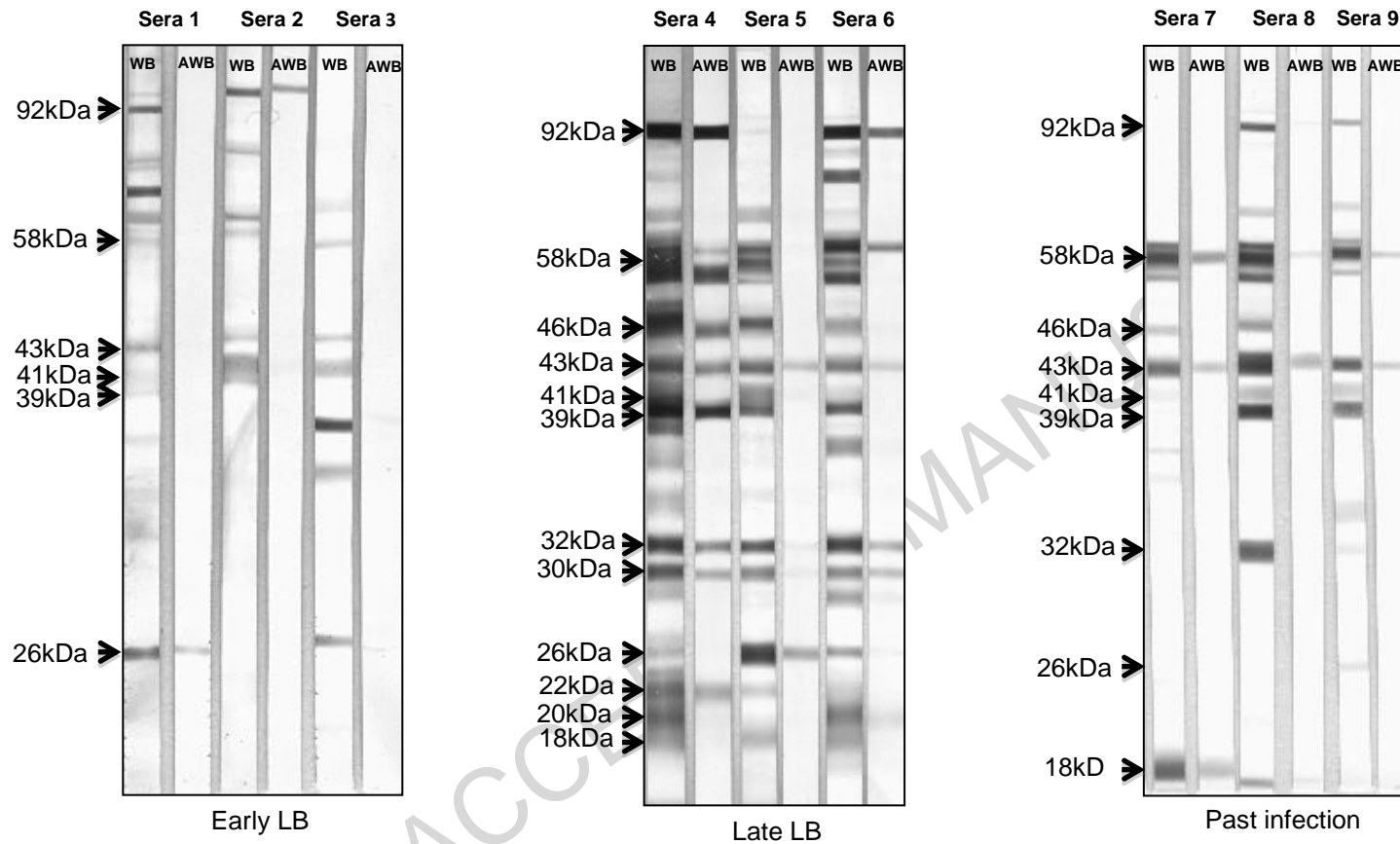


Figure 2: Western blot (WB) and avidity Western blot (AWB) profiles of three patients in the evaluation sera panel with early LB (symptoms <6 month onset, sera 1-3), three patients with late LB (symptoms >6 month onset, sera 4-6) and three patients with past infection (symptomatic and seropositive but not clinically deemed to have LB, sera 7-9).

Avidity Western blot evaluation

There were visible differences in the Western blot and avidity Western blot banding profiles for the majority of sera from patients with early and late LB and past infection (Figure 2).

The number of proteins detected by antibodies in the Western blot (total IgG antibodies) and by high and low avidity antibodies (avidity Western blot) increased between early and late infection groups before moderately decreasing in past infections (Figure 3).

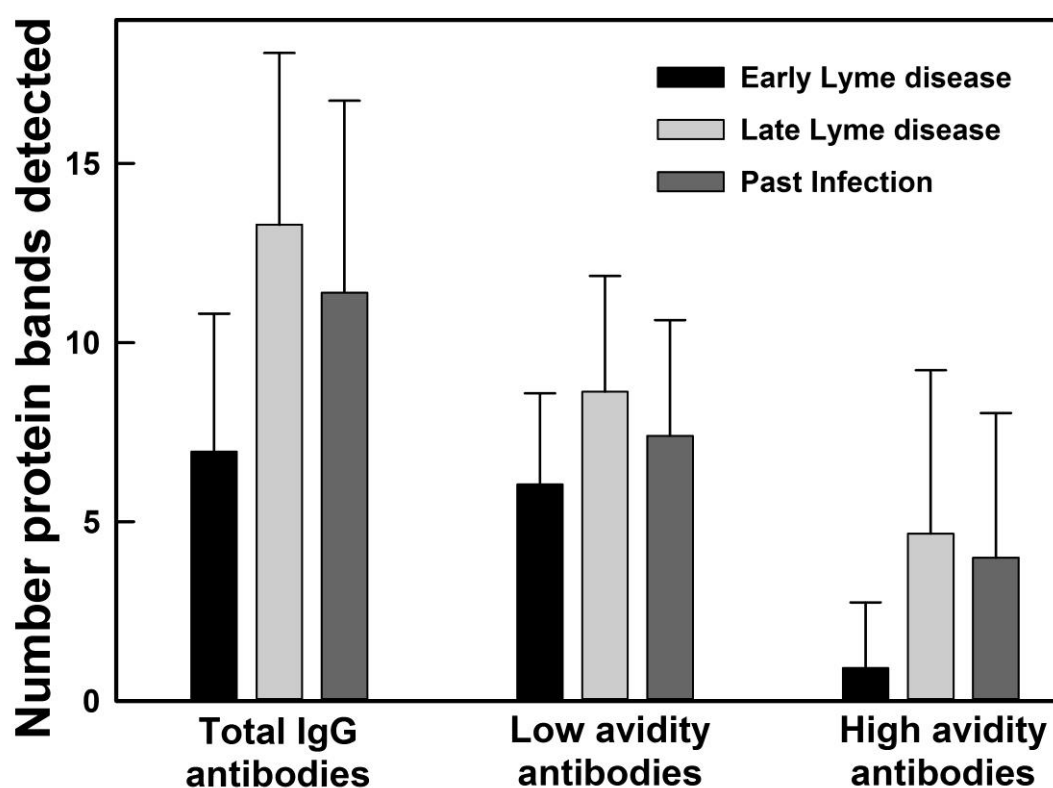


Figure 3: Number of proteins detected by antibodies in the Western blot (total IgG antibodies) and by low and high avidity antibodies (avidity Western blot) in sera from patients with early (n = 26), late (n = 24) and past (n = 25) LB. Data are presented as means \pm SD.

These differences (total IgG antibodies, high avidity antibodies, low avidity antibodies) were significant between the early and late groups ($P < 0.001$, $P = 0.005$, $P < 0.001$, Mann-

Whitney U Test, respectively) and early and past infection groups (when comparing the number of proteins detected by total and low avidity antibodies, $P = 0.002$ and $P < 0.001$ Mann-Whitney U Test, respectively). However, no significant differences were observed between the late and past infection groups.

When PLDA was used to compare the Western blot and avidity Western blot results from all three patient groups in the evaluation panel, 42/75 (56%) of the original patients were correctly classified into their patient groups when the results were cross validated. The prediction accuracy was considerably higher when only two groups were compared at any time: early versus late LB (38/50, 76%); early LB versus past infection (40/51, 78%) and late LB versus past infection (34/49, 69%).

Western blot/ avidity Western blot and PLDA validation

When the PLDA model trained on the data from all 3 groups in the evaluation panel was used to predict disease status for the 20 sera in the CDC sera panel, based on the Western blot and avidity Western blot results, 18/20 (90%) were correctly classified, with only one patient with early neurological LB and one patient with late LB (arthritis) falsely predicted (Table 1). The 2-group models, trained on the early LB versus past infection and late LB versus past infection data, correctly classified the same 18 samples when used on the early and late LB sera, respectively (Table 1).

Model		3-group model (n=20)			Early vs. Past model (n=12)			Late vs. Past model (n=8)		
Model-predicted group		Early LB	Late LB	Past	Early LB	Late LB	Past	Early LB	Late LB	Past
Clinical picture	Early LB (n=12)	11 (55%)	1 (5%)	0	11 (92%)	-	1 (8%)	-	-	-
	Late LB (n=8)	1 (5%)	7 (35%)	0	-	-	-	-	7 (88%)	1 (12%)

Table 1 – Stage of infection predicted by Western blot/ avidity Western blot and PLDA for CDC sera and comparison with clinical picture

Western blot/ avidity Western blot and PLDA testing with routine clinical samples

Sera from 112 patients tested seropositive by the routine testing protocol at NLBTL were also tested by Western blot and avidity Western blot. Of these, 29 patients had symptoms for less than six months, and so were analysed with the early LB versus past infection PLDA model. The clinical picture and PLDA prediction results were compatible for 21/29 (72%) of these patients (Table 2). The remaining 83 patients had symptoms for more than six months, and so were analysed with the late LB versus past infection PLDA model. This time, the clinical picture information and PLDA predictions were compatible for only 32/83 (39%) of these patients (Table 2). The PLDA model trained on the data from all 3 groups was not utilised with this data set.

Model		Early vs. Past model (n=29)			Late vs. Past model (n=83)		
Model-predicted group		Early LB (n=14)	Late LB (n=0)	Past (n=15)	Early LB (n=0)	Late LB (n=58)	Past (n=25)
Clinical picture	Early LB (n=16)	11(38%)	-	5(17%)	-	-	-
	Late LB (n=13)	-	-	-	-	10(12%)	3(4%)
	Past infection (n=83)	3(10%)	-	10(34%)	-	48(58%)	22(27%)

Table 2 – Stage of infection predicted by Western blot/ avidity Western blot and PLDA for NLBTL routine sera and comparison with clinical picture

Discussion

Current serological methods cannot readily distinguish patients actively infected with *B. burgdorferi* sensu lato from those who have been infected in the past (Moore et al., 2016). This is especially problematic for seropositive patients with persisting non-specific symptoms. Some patients with LB may not be given the necessary antibiotic treatment as they do not fulfil the strict LB case definitions, whereas other patients may be incorrectly labelled as LB, unnecessarily treated with antibiotics and not investigated further for alternative causes of their symptoms. Therefore, the ability to distinguish active from past infection could help prevent inappropriate patient management. This study reports the development of an avidity Western blot for the serological testing of LB patients and assesses its potential to address this important clinical need.

While antibodies to certain *B. burgdorferi* s.l. proteins are detected at different stages of the infection, Western blot profiles alone are not sufficient to differentiate patients with late LB and past infection with any confidence. The avidity Western blot approach was therefore a logical step, but due to the vast array of information obtained from the avidity Western blot and Western blot profiles from each patient it was necessary to statistically compare the complex antibody-protein binding patterns obtained from testing sera from patients with different stages of LB. When PLDA was used to compare the Western blot and avidity Western blot results from all 3 patient groups in the evaluation panel (early LB, late LB and past infection), 56% of patients were predicted to be correctly classified in their patient

groups. When only two groups were compared at any one time i.e. early with late LB or past infection and late LB with past infection, the correct prediction results were much higher (69 to 78%). Whilst these prediction rates were good, the lack of well-characterised patients with late LB and past infection in the evaluation/training panel may have prevented the prediction results from being even higher. Unfortunately, there is poor availability of well-characterised sera panels, especially those with late LB or past infection in the UK, or indeed, Europe. If the only patients to be included in the late LB group within the evaluation panel were to be those that fitted the European case definitions (Stanek et al., 2011) the numbers would be too small to allow group comparisons to be made as only 6/24 patients would have been included (3 with acrodermatitis chronic atrophicans and 3 with long-standing borrelia lymphocytoma/erythema migrans), although reassuringly, 5/6 were predicted by PLDA to have late Lyme disease. As a result, this study had to rely on questionnaire responses i.e. if symptoms were for more than 6 months and the clinician had determined the patient had LB. As many of the patients in this group lacked specific signs of LB it is possible that these patients had indeed had infection in the past as predicted by the PLDA, and that their current symptoms were not due to LB, as determined by their clinician. Likewise, clinical acumen had to be relied upon with the patients assigned to the past infection evaluation panel as those determined by the clinician as not having LB despite having positive serology.

When the Western blot and avidity Western blot results from the CDC sera were analysed with the 3-group and 2-group models respectively, 90% of patients with early and late LB were predicted correctly. These results certainly appear to validate the use of the Western blot/avidity Western blot and PLDA approach with this high confidence dataset, which consisted of sera from the CDC Lyme disease serum repository from well-documented patients with different stages of LB, (Molins et al., 2014). The Western blot/ avidity Western

blot and PLDA approach was also encouraging when tested against a lower clinical confidence data set consisting of single and multiple sera from patients whose clinical details were less defined, and more representative of those routinely seen in the diagnostic laboratory. As it would be of most use to be able to distinguish past infection from early or late LB (i.e. active infection) in the routine setting, it was proposed that one of the PLDA models based on the two group comparisons, with a higher predictive accuracy, should be used instead of testing all patient results with the single PLDA model based on the three group comparison. Therefore, the Western blot and avidity Western blot results for patients presenting with symptoms for less than 6 months were tested against the early LB versus past infection PLDA model. Likewise, for patients presenting with symptoms for more than 6 months, the late LB versus past infection PLDA model was used. Using these 2-group models, the PLDA predicted that 40 patients had evidence of past rather than active (early or late) infection (Table 2). Although actual group membership was difficult to determine with absolute confidence clinically, the prediction of past infection was compatible with the clinical picture for 32 (80%) of these patients.

The results from this study did not allow us to determine how long it takes for an avidity profile to change from late LB to past infection. Whilst 72% of the early vs. past infection model predictions were compatible with the clinical picture, when the routine sera were analysed with the late versus past infection PLDA model, there was only 39% agreement with the clinical picture (Table 2). The majority (48/51) of these discrepant patients were predicted by the PLDA models to have late LB whereas the clinical details were indicative of past infection. It is possible that in these patients the clinical picture of past infection was more accurate than the model predictions of late LB and that the model predictions were influenced by persisting high avidity antibodies. It is not clear from the literature whether

antibodies that have undergone avidity maturation are maintained over time if there is no antigenic re-stimulation. High avidity antibodies could be produced following clearance of the infecting organism but may decay relatively fast thereafter. On the other hand, there may be a significant lag period so that high avidity antibodies persist. It could be postulated that this may be influenced by how early antibiotic treatment is given, which would explain the persistence of high avidity antibodies in the late LB patient group. We also cannot rule out that these patients have been re-exposed at some point during their sampling period, enabling the specific immune response to be pump-primed, thus maintaining the avidity profile of late LB. The controversial concept of persisting infection, or *B. burgdorferi* s.l. proteins persisting in these patients (Steere, 2012) also has to be considered.

Whilst lysate Western blot is an ideal research tool to examine the antibody avidity response to a wide range of borrelia antigens, issues with Western blot reproducibility and standardisation (especially with in-house assays) (Robertson et al., 2000) have led to the use of commercial immunoblots, utilising purified and recombinant antigens, in routine clinical laboratory practice. To simplify and standardise this complex Western blot/avidity Western blot with PLDA approach, proteins identified by the PLDA as the most important for determining the stage of infection (i.e. predictor variables)(data not shown) could be investigated for use in a more user-friendly and standardised alternative avidity assay format in future work

In conclusion, we have developed an IgG avidity Western blot which, when combined with Western blot and PLDA, shows exciting potential for being able to predict LB disease stage, and hence improve patient management in the future. The technique has demonstrated promising results for distinguishing patients with early LB from those who have been infected with *B. burgdorferi* s.l. in the past, and to a lesser extent, can distinguish some

patients with past infection from those with late LB. The strong potential of this approach, especially when no alternative methods are currently available, warrants further research to confirm applicability in the clinical setting. A large-scale longitudinal clinical study of LB patients would provide the necessary sera and clinical details to assess the timescale of borrelia antibody avidity. Likewise, an expanded panel of sera from well-defined patient populations (including those with past infection) from both low and high incidence European countries would undoubtedly produce a more comprehensive picture and allow the PLDA models to be updated, refined and further verified and enable the sensitivity and specificity of this novel Western blot/ avidity Western blot with PLDA approach to be assessed.

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Highlights

- Serological methods cannot currently distinguish active from past Lyme borreliosis
- We report the first use of an avidity Western blot for this purpose.
- When analysed with PLDA, some patients with past infection were identified
- Our test may predict disease stage in some patients and improve their management
- An expanded sera panel from well defined patients is required for further validation

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