Lyme borreliosis: The diagnostic value of laboratory assays

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Abstract

Lyme borreliosis is caused by an infection with the spirochete *Borrelia burgdorferi*. As the symptoms associated with Lyme disease are unspecific clinical diagnosis of the disease is difficult. A two-tier testing method, consisting of a enzyme immunoassay followed by an immunoblot in case of positive or equivocal results, is currently practiced to demonstrate the presence of the bacteria in the serum of a potential patient. However, the sensitivity and specificity of laboratorial tests are often inadequate. In this review the reliability of a number of laboratorial assays is evaluated. Among the immunoassays appraised here, the ELISA using a synthetic peptide derived from the antigenic VlsE protein of *Borrelia* appeared to be the most accurate first tier test. Differences between confirmatory Western blotting assays seem to be minor. PCR in skin biopsies or synovial specimens may provide an additional contribution to diagnosis. A positive antibody index and pleocytosis are indicative of Lyme neuroborreliosis. Still, due to the suboptimal performance of any laboratorial test method diagnosis of Lyme borreliosis should be based on a combination of the clinical manifestation, laboratory tests and the a priori chance of Lyme disease.

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Introduction

Lyme borreliosis (LB), or Lyme disease, is currently the most common tick-borne disease in Europe (Satz 2002). The illness is caused by an infection with bacteria of the *Borrelia burgdorferi* sensu lato complex. The pathogenic importance of various *Borrelia burgdorferi* genospecies is related to their geographical distribution pattern (Parola & Raoult 2001). While in North America *B. burgdorferi* sensu stricto is the major Lyme borreliosis inducing agent, in Europe *B. garinii*, *B. afzelii*, *B. spielmanii* as well as *B. burgdorferi* sensu stricto are capable of causing the disease (Steere 2001, Hengge *et al.* 2003, Wilske *et al*. 2007).

Borrelia bacteria belong to the phylum of spirochetes. They are thus characterized by helically shaped cells, Gram-negativity and the presence of multiple periplasmic flagella. Specific for *Borrelia* is that transmission of the bacteria between vertebrates is mediated through arthropods (Barbour & Hayes 1986). Although many animals can act as a host (Hengge *et al.* 2003), rodents form the major natural reservoir for the bacteria (Nicolle 1927). *Borrelia* species are depending on the presence of a host, as they are unable to proliferate outside it (Barbour & Hayes 1986).

Ticks of the *Ixodes* species form the primary vector of *Borrelia burgdorferi.* The life-cycle of the ticks consists of three stages, namely larval, nymphal and adult. The *Ixodes* ticks become infected with *Borrelia* by feeding on the blood of a host. Though infection may occur during any life stage, nymphal ticks are likeliest to transmit the bacteria to humans, as this life stage prevails during spring and summer. Moreover, as nymphal ticks are relatively small they often remain unnoticed (Hengge *et al.* 2003).

Lyme borreliosis can affect a variety of tissues and the clinical manifestation alters during progression of the infection, as is shown in Table 1. The disease can be classified as early or late and is subdivided in three stages with multifarious symptoms. The early localized stage I infection of the first days or weeks after the tick-bite is usually accompanied by erythema migrans (EM) around the site of infection. During the second stage the infection becomes systemic and many different symptoms may occur. Six months after the tick bite the disease enters stage III, while the occurrence of systemic symptoms continues (Nau *et al.* 2009). After treatment of the infection has been completed patients may still experience signs of LB such as fatigability, impaired performance and an impaired ability to concentrate (Krupp *et al.* 1991). In these cases of 'chronic Lyme disease' or 'post Lyme syndrome' studies are incapable of demonstrating the presence of *B. burgdorferi* (Auwaerter 2007). The existence of chronic Lyme disease as a genuine medical condition is therefore highly controversial (Tonks 2007).

Table 1 Clinical manifestation of Lyme borreliosis (Nau *et al.* 2009)

Stage of infection	Symptoms
Stage I	Erythema migrans around the site of infection
Stage II	Meningoradiculitis, meningitis, peripheral facial palsy, encephalitis, myelitis, cerebral arteritis, multiple erythemas, arthritis, myalgia, borrelial lynphocytoma, myositis, myo- or pericarditis, iritis
Stage III	Encephalitis or encephalomyelitis, cerebral arteritis, polyneuropathy, mono- or oligoarthritis, acrodermatitis chronica atrophicans

Although many organs may be affected by Lyme borreliosis, the brain generally experiences the most persistent infection (Barbour & Hayes 1986). In case of such neurological involvement the infection is referred to as Lyme neuroborreliosis (LNB) (Hildebrand 2009). Notably, *Borrelia garinii* is often found in association with the occurrence of neuroborreliosis (Wilske *et al.* 1996).

Lyme borreliosis is usually treated by means of the administration of antibiotics. This has shown to be beneficial for the duration of the clinical course and prevents the development of complications (Kaiser 2004). Antibiotic treatment has also been proved to successfully contribute to the recovery from stage II neuroborreliosis. In case of stage III LNB the neurological deficits will clear less rapidly and a higher chance of residual damage exists (Kaiser 2004). However, Antibiotic therapy of over 28 days is discouraged, since long-term usage of these substances increases the chance of side-effects (Nau *et al.* 2009).

Because the clinical manifestation of LB is complex and many of the symptoms are not specific for Lyme borreliosis, diagnosis of the disease is critical (Hofmann 1996). It has been suggested that the best procedure for diagnosis is based on a combination of the clinical expression of symptoms, tick-exposure, epidemiology and IgG and IgM antibody serology. Currently a two-tier approach is usually practiced in order to detect the presence of *B. burgdorferi*, consisting of a sensitive enzyme immunoassay (EIA), followed by a confirmatory immunoblot in case of positive or equivocal test results (Tjernberg *et al.* 2007, Hildebrand *et al.* 2009). However, the sensitivity and specificity of these test might not be sufficient to obtain accurate results, especially in the early stages of infection (Hofmann 1996, Nau *et al.* 2009). Polymerase Chain Reaction (PCR) or cultivation of the bacteria could provide alternative diagnostic methods (Nau *et al.* 2009). Still, these procedures are also associated with certain difficulties (Hofmann 1996, Nau *et al.* 2009).

In this review several diagnostic methods will be evaluated in order to identify appropriate techniques for the detection of an infection with *Borrelia burgdorferi.*

Limitations in diagnosis of Lyme disease

As mentioned above, a number of laboratorial techniques have the potential to be used for demonstration of an infection with *B. burgdorferi*. Some diagnostic methods, such as cultivation, microscopy and PCR show the presence of the spirochete directly, while serological and blotting methods are indirect as they identify the IgM or IgG antibodies that are produced against the pathogen. Each technique has its own combination of sensitivity and specificity for *Borrelia*, depending on the limitations by which it is affected. An overview of factors that may impair the accuracy of diagnostic techniques is given here.

Low numbers of bacteria
The detection of *Borrelia burgdorferi* in tissues and body fluids of patients is impaired by the low numbers of bacteria that are present. Consequently, the sensitivities of PCR and microscopy as diagnostic techniques are reduced (Aguero-Rosenfeld *et al.* 2005). The amount of material that is available for PCR diagnosis may be decreased even further by inadequate methods for sampling, storage, transportation and preparation of bacterial DNA (Aguero-Rosenfeld *et al.* 2005). Moreover, although isolation of *Borrelia burgdorferi* from EM-sites and the inflamed part of acrodermatitis chronica atrophicans (ACA) is possible, the isolation of bacteria for culturing from other tissues, blood and body fluids is generally unsuccessful (Lakos 2009). This is especially problematic because in patients with extracutaneous Lyme borreliosis clinical diagnosis is generally difficult. The reliance on laboratorial methods is therefore more important when cutaneous symptoms are absent (Nadelman & Wormser 1998).

Variations in antigen expression
Another difficulty that is associated with confirmation of the presence of *Borrelia* bacteria in Lyme patients is caused by the heterogeneity of the pathogens. Differential expression of bacterial antigens between *B. burgdorferi* species or even between strains of the same species makes it difficult to point out antibodies or PCR primers which ought to be used for diagnosis (Wilske *et al.* 2007). Because a variety of antibodies are currently used for diagnosis (Aguero-Rosenfeld *et al.* 2005), the sensitivity and specificity of the marketed serological tests are diverse (Lakos 2009).

Moreover, the antigens that are expressed by *Borrelia burgdorferi* alter in response to changing environmental factors, such as temperature, pH and cell density (Mueller *et al.* 2006). The antigen expression of *Borrelia* is therefore different in vectors and hosts (Ohnishi *et al.* 2001). The bacteria are also known to reduce gene expression after entering a host (Liang *et al*. 2002). Especially in the early stages of LB diagnosis is impaired by the unpredictability of antigen expression (Peterson *et al.* 2008).

Cross-reactivity
Tests that are used to prove the presence of IgM or IgG antibodies might yield false-positive results by cross-reacting with non-borrelial antigens. Especially in IgM antibody tests there is high probability for non-specific reactions (Lakos 2009). The low specificity of antibody test in patients that suffer from other diseases is caused by the fact that immunological antigens of *Borrelia burgdorferi* share similarities with other bacteria and viruses (Bruckbauer *et al.* 1992, Brown *et al.* 1999).

Delayed immunological response
As antibody production against *Borrelia* develops during the third to sixth week after infection, serological diagnostic methods for Lyme are impaired during the first weeks (Lakos 2009). IgM antibodies appear 3-6 weeks after the beginning of infection, while IgG antibodies are produced 6 weeks to 3 months after the start of infection (Dressler *et al.* 1993). Antibody concentrations increase with progression of the disease (Wilske *et al.* 2007). Moreover, the anti-borrelial antibodies remain present in the body even after bacterial clearance. It is therefore difficult to distinguish an active infection from a past infection.

Interpretation of data
Although Western blotting is know to be a rather specific method for detecting *Borrelia*, standardization of this method is difficult so that controversy concerning the interpretation of the data exists (Rauer *et al*. 1998).

Borrelial antigens

As both the Western blot and the ELISA, which presently comprise the standard procedure for detection of *Borrelia* (Hildebrand *et al.* 2009), are based on the demonstration of antibodies against *Borrelia* it is important to determine which borrelial antigens induce a humoral response in Lyme disease patients. The identification of such antigens is impaired by the fact that different antigens are expressed by various species and strains and expression of antigens can change during transmission from vector to host (Aguero-Rosenfeld *et al.* 2005). Nevertheless a number of immunogenic antigens have been identified.

Flagellin A
The borrelial flagellar protein Flagellin A (FlaA) is an immunodominant antigen which is especially important during the early stages of infection (Dressler *et al.* 1993). FlaA is a well conserved protein among spirochetes (Ge *et al.* 1998). Nevertheless, its presence in *B. burgdorferi* is only minor in comparison with the expression of flagellin B (Panelius 2002).

Flagellin B
Flagellin B, otherwise known as flagellin, FlaB or P41, composes the major periplasmic flagellar filament. The protein has been recognized as an immunodominant borrelial antigen (Hansen 1994) and even in the early stages of infection strong antibody responses are elicited against it (Dressler *et al.* 1993). Still, antigens from other bacteria as well as mammalian tissues have been found to be similar to flagellin B, so the specificity of diagnostic tests targeting this protein is reduced. The internal portion of the protein which contains the genus-specific domain, is however less cross-reactive than the whole protein (Luft *et al.* 1993).

BmpA
BmpA, also known as P39, is a chromosomally encoded immunogenic lipoprotein (Fraser *et al.* 1997). The *bmp* genes by which it is encoded are conserved in the DNA sequence of all *B. burgdorferi* sensu lato species (Gorbacheva *et al.* 2000).

DbpA
Decorin binding protein A (DbpA), which is also referred to as Osp17 or P18, is associated with the binding of *Borrelia* to the hosts collagen-associated proteoglycan decorin. Interspecies differences in the amino-acid sequences of DpbA exist. DbpA is produced by *Borrelia* both *in vitro* and *in vivo* and expression of the protein is temperature-regulated (Aguero-Rosenfeld *et al.* 2005).

IR6-region of the VlsE
The Vmp-like sequence expressed (VlsE) protein is a immunogenic plasmid encoded lipoprotein (Zhang *et al.* 1997). The gene consists of variable and invariable domains. One specific variable domain which is known as the sixth invariable region (IR6) lies in the central region of the VlsE protein. This region of the VlsE has been found to be expressed early after infection, is highly immunogenic and is conserved among all *Borrelia* species (Liang *et al.* 1999).

BBK32
BBK32, or P35, is a fibronectin binding protein which is expressed during early stages of infection. It is up-regulated during transmission from the tick to mammalian hosts after which its expression is reduced once more (Fikrig *et al.* 2000).

RevA
RevA has also been identified as a fibronectin binding protein. Though humans are often exposed to RevA during an infection with *Borrelia* the protein can not be detected in nymphal ticks (Gilmore *et al.* 2001, Brissette *et al.* 2009).

P83/100
The chromosomally encoded protein P83/100 is associated with the flagella or the periplasmic cylinder (Perng *et al.* 1991, Eiffert *et al.* 1992, Luft *et al.* 1992a). The genes coding for P83/100 are highly identical among different species of *Borrelia burgdorferi* (Luft *et al.* 1992b) and are also very specific for *B. burgdorferi* (Bruckbauer *et al.* 1992). P83/100 is usually expressed in very low amounts (Rössler *et al.* 1995).

OspC
Outer surface protein C (OspC) is a plasmid encoded protein which is first expressed during the ticks feeding on the host (Padula *et al.* 1994). The protein is associated with the establishment of an infection in the host (Stewart *et al.* 2006). It is therefore an important antigen particularly early after infection with *Borrelia* (Stevenson *et al.* 1995). Once inside the host an immune response is elicited by the binding of OspC to Toll-like receptor 2 (TLR2) (Brightbill *et al.* 1999). OspC is present in both *B. burgdorferi* sensu stricto and *B. garinii* as well as *B. afzelii* (Palenius *et al.* 2002), although some heterogeneity of this protein between *B. burgdorferi* sensu lato species and within bacteria of the same species exists (Jauris-Heipke *et al.* 1993). There is however at least 70% homology between the OspC genotypes (Wang *et al.* 1999).

Table 2 Borrelial surface proteins and their immunoreactivity with human sera (Nowalk *et al.* 2006)

Protein	EL	ED	LD
RevA (BBM27, BBP27)		+	
ErpA/I/N (BBL39, BBP38, OspE paralog, $_{Bb}$CRASP-5)		+	
P66 (BB0603)	-/+	+	+
ErpP (BBN38, OspE paralog, $_{Bb}$CRASP-3)		+	+
Lipoprotein LA 7 (BB0365)		+	+
BB0323		+	+
BBA64 (P35)		+	
BBI36/38			
BmpA (BB0383, P39)		+	+
FlaB (BB0147)	+	+	+
ErpB/J/O (BBL40, BBP39, ElpB1)		+	+
FlhF (BB0270)		+	+
BBA66		+	+
OppA I, II, and IV (BB0328, BB0329, BBB16)	-/+	+	+
OppA III (BB0330)		+	+
OspA (BBA15)			+
Hsp90 (BB0560)		+	+
P83/100 (BB0744)		+	+
FtsZ (BB0299)		+	+
DbpA (BBA24)		+	+
OspC (BBB19)		+	+

-/+, weakly immunoreactive; +, strongly immunoreactive.
EL, early localized; ED, early disseminated; LD, late disseminated.

Other antigens
Furthermore, many other immunogenic membrane proteins have been identified. A selection of these proteins is enlisted in Table 2.

Evaluation of commercially available test kits

Immunoassays
The current first-tier method that is used for laboratorial diagnosis of Lyme disease is an EIA to detect antigens against *B. burgdorferi*. An enzyme-linked immunosorbent assay (ELISA) is the most commonly used enzyme immunoassay because of its advantages with respect to ease of testing, objective generation of values that correlate with the quantity of antibodies present and the possibility to automate the procedure (Aguero-Rosenfeld *et al.* 2005). An enzyme-linked immunofluorescent assay (ELFA), indirect immunofluorescent-antibody assay (IFA) or other serological techniques may also be used as a first-line test to detect anti-borrelial antibodies (Aguero-Rosenfeld *et al.* 2005).

Table 3 Commercial immunoassay test kits and their characteristics

Test	Antigen specifics	References
Synthetic peptides		
Quick ELISA C6 *Borrelia* assay (Immunetics)	Synthetic C6 peptide, amino acid sequence derived from the VlsE protein from *B. burgdorferi*, strain B31 The test does not discriminate between IgM and IgG antibodies	Marangoni *et al.* 2005a, Matthys *et al.* 2007, Tjernberg *et al.* 2007, Wormser *et al.* 2008
Recombinant antigen		
Recomwell *Borrelia* ELISA test (Mikrogen)	Recombinant *B. burgdorferi* sensu lato antigens	Marangoni *et al.* 2005a
LIAISON *Borrelia* IgM (310890) chemiluminescence immunoassay (DiaSorin)	Recombinant OspC from *Borrelia afzelii*, strain pKo	Petersen *et al.* 2008
LIAISON *Borrelia* IgG (310880) chemiluminescence immunoassay (DiaSorin)	Recombinant VlsE from *B. garinii*, strain pBi	Marangoni *et al.* 2007, Petersen *et al.* 2008
Recombinant/native antigen		
Enzygnost Lyme link VlsE/IgG (DADE Behring)	Mix of native *Borrelia*-antigens from the pKo strain of *B. afzelii* and recombinant VlsE obtained from *B. burgdorferi* sensu stricto, *B. garinii* and B. *afzelii*	Marangoni *et al.* 2007, Matthys *et al.* 2007
Native antigen		
Enzygnost Borreliosis test (DADE Behring)	Detergent extract of *B. afzelii*, strain PKo	Marangoni *et al.* 2005a, Matthys *et al.* 2007
Virotech *Borrelia burgdorferi* ELISA (Genzyme)	Purified flagella and flagellar complexes	Tjernberg *et al.* 2007
IDEIA *Borrelia burgdorferi* (DakoCytomation)	Purified flagella	Petersen *et al.* 2008

While different borrelial antigens are expressed under varying circumstances, the selection of antigens that are used for confirmation of the presence of IgM and IgG antibodies against *Borrelia* plays an important role in determining the sensitivity and specificity of the test. Antigens that are currently used in serological testing can be subdivided into three classes: native antigens, recombinant antigens and synthetically produced peptides (Aguero-Rosenfeld *et al.* 2005). Table 3 provides an overview of a number of commercially available test kits and specific characteristics of the antigens on which they are based. Various studies have been dedicated to the validation of commercially available *Borrelia burgdorferi* immunoassays. A selection of these researches will be discussed in the next section.

The Quick ELISA C6 *Borrelia* kit (Immunetics), the Virotech *Borrelia burgdorferi* ELISA (Genzyme) and the LIAISON *Borrelia* IgM (310890) IgG (310880) chemiluminescence immunoassay (DiaSorin) were tested for their sensitivity and specificity in Swedish test subjects with symptoms of Lyme disease, Lyme neuroborreliosis and a control group of random blood donors. Cross-reactivity with serum from patients that were tested positive for Epstein-Barr virus, rheumatoid arthritis or syphilis infection was also examined. The IgM and IgG results were combined and classified either positive, negative or equivocal. Results of this study are shown in Table 4. These data indicate that the assay based on the synthetic C6 peptide functions at least as well as the Virotech *Borrelia burgdorferi* ELISA IgG/IgM test and the LIAISON *Borrelia* IgM/IgG chemiluminescence immunoassay. The Immunetics Quick ELISA C6 *Borrelia* assay therefore seems a suitable first-tier testing method (Tjernberg *et al.* 2007).

Table 4 Test results for LB diagnostic techniques (Tjernberg *et al.* 2007)

Study group	Test result	IQC6 (%)	VT ELISA (%)	Li CLIA (%)
LB symptoms	+	49	51	49
	+/-	2	10	5
LNB	+	88	88	80
	+/-	0	8	4
Cross-reactivity	+	27	36	11
	+/-	2	18	11
Blood donors	+	8	14	12
	+/-	0	10	7

IQC6, Immunetics Quick ELISA C6 *Borrelia* assay kit; VT ELISA, Virotech *Borrelia burgdorferi* ELISA IgG/IgM test kit; Li CLIA, LIAISON *Borrelia* IgM (310890) IgG (310880) chemiluminescence immunoassay; LB, Lyme borreliosis; LNB, Lyme neuroborreliosis; +/-, equivocal; +, positive

The Quick ELISA C6 *Borrelia* assay (Immunetics) was also compared with the RecomWell *Borrelia* ELISA test (Mikrogen) and the Enzygnost Borreliosis test (DADE Behring). For determination of the sensitivities of these tests sera from Italian patients with culture-confirmed Lyme borreliosis were used. The specificity of the tests was examined using sera from both healthy test subjects and a group of patients with infections other than Lyme borreliosis that could induce cross-reactivity. The results that were found are shown in Table 5. Performances of the Quick ELISA C6 assay as well as the RecomWell *Borrelia* assay were ratified as acceptable for the use of clinical diagnosis (Marangoni *et al.* 2005a).

Table 5 Sensitivity and specificity of different ELISAs (Marangoni *et al.* 2005a)							
Testing method	RecomWell Borrelia			Enzygnost Borreliosis			IQC6
Antibodies	IgG	IgM	Overall	IgG	IgM	Overall	Overall
Sensitivity (%)	57.9	55.7	73.7	36.8	70.5	77.9	62.1
Specificity (%)	97.1	98.9	96.0	90.1	92.3	82.5	96.7

IQC6, Immunetics Quick ELISA *Borrelia* C6 assay

The Quick ELISA C6 (Immunetics), VIDAS Lyme (BioMérieux), Enzygnost Borreliosis IgM and IgG (DADE Behring), Enzygnost Lyme link VlsE/IgG (DADE Behring) and anti-*Borrelia* IgM and IgG (Eurimmun) were compared in Lyme borreliosis patients with various symptoms. The lowest sensitivity (94.5%) and specificity (66%) were found for the VIDAS Lyme. The Quick ELISA C6 had the highest sensitivity and specificity of 98.6% and 96% respectively. Overall, the Quick ELISA C6 also provided the best positive (97.3%) and negative (98.0%) predictive value. The Quick ELISA assay thus seems a more appropriate test for diagnosis of infection with *B. burgdorferi* than the other four screening assays (Matthys *et al.* 2007).

In North America the possibility of using the C6 Lyme ELISA kit (Immunetics) as a replacement for two-tiered testing was evaluated. The C6 Lyme test kit was therefore compared to the two-tiered method using an IgG/IgM ELISA kit (Wampole Laboratories), followed by Lyme IgG and IgM immunoblots (MarDx/Trinity Biotech), all of which use a sonicated whole-cell preparation of *B. burgdorferi* B31. A sensitivity of 69.5% was found for the C6 ELISA against 38.9% sensitivity for the two-tiered testing method among patients with culture-confirmed Lyme borreliosis. The results seem to confirm that a C6 peptide based ELISA is indeed a suitable alternative for the two-tiered testing method. However, the authors speculated that the performance of the two-tiered test was impaired by the low sensitivity of the immunoblot test kit that was employed in this study (Wormser *et al.* 2008).

The performances of the Enzygnost Lyme link VlsE/IgG kit (DADE Behring) and the LIAISON *Borrelia* IgG test kit (DiaSorin) were examined using sera from 66 patients with PCR confirmed *B. afzelii* infection. While the Enzygnost VlsE/IgG identified 27 serum samples as reactive, 10 as border line and 29 as negative, the LIAISON *Borrelia* IgG only scored 21 sera as positive, 5 as border line and 40 as negative. Thus, the Enzygnost Lyme link VlsE/IgG assay is more sensitive than the LIAISON *Borrelia* IgG test (Marangoni *et al.* 2007).

Test results of the IDEIA *Borrelia burgdorferi* test (DakoCytomation) and the LIAISON chemiluminescence IgM/IgG immunoassay (Diasorin) were compared using unselected sera from general health practices in Denmark. In case of discrepancy between the two tests the serum sample was additionally analyzed by an Ecoline blot (Virotech). Out of the 357 serum samples, 308 negative and 3 positive samples were found with concordant results of the IgM test assays. Compared with the line blot 21 false-positive IgM results were yielded by the IDEIA assay, while only 1 false-positive result was produced by the LIAISON test. For the IgG tests 329 results were similar between the two tests, of which 1 was positive and 328 were negative. 4 false-positive results were produced by the LIAISON, although this method also detected 9 positive results that were missed by the IDEIA test. These results indicate that the recombinant VlsE antigen-based test is a more reliable method for the diagnosis of Lyme disease than an assay that is based on flagellar proteins (Petersen *et al.* 2008).

These results discussed above consistently indicate that the Immunetics Quick C6 ELISA is a relatively reliable commercial immunoassay for the demonstration of antibodies against *Borrelia*. The recomwell *Borrelia* ELISA, Virotech *Borrelia burgdorferi* ELISA or LIAISON chemiluminescence IgM/IgG immunoassay might also be suitable as a first-tier test assay, but the reliability of these tests has not been examined as extensively.

Immunoblots
The currently used second-tier test consists of an immunoblot. This test is especially performed for its specificity, as it is possible to discriminate between different proteins (Aguero-Rosenfeld *et al.* 2005). There are Western blots which are based on whole cell lysates or recombinant antigens and line blots which use recombinant antigens which are not denaturated by electrophoresis (Hunfeld & Kraiczy 2009). Performances of a few commercially available Western blots kits will be evaluated. Features of these immunoblot kits are shown in Table 6.

Table 6 Commercial immunoblot test kits and their characteristics

Test	Antigen specifics	References
Recombinant antigens		
RecomBlot *Borrelia* (Mikrogen)	Recombinant P100, P41, P39, OspA and P18 derived from *B. afzelii*, OspC from *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* and the internal part of P41 derived from *B. afzelii* and *B. garinii*	Marangoni *et al.* 2005a
Native/recombinant antigens		
Euroline-Western blot (Euroimmun)	Proteins from *B. afzelii* plus a membrane chip coated with recombinant VlsE antigen	Marangoni *et al.* 2005b
Native antigens		
B. burgdorferi Marblot (MarDX)	*B. burgdorferi* strain B31 proteins	Mogilyansky *et al.* 2004
BBI Lyme WB	*B. burgdorferi* sensu stricto proteins of strain 2591	Mogilyansky *et al.* 2004
Borrelia Virablot (Viramed)	Proteins from *B. burgdorferi* sensu stricto and *Borrelia afzelii*	Mogilyansky *et al.* 2004
Borrelia burgdorferi EcoBlot (Genzyme Virotech)	*B. burgdorferi* sensu stricto proteins of strain 2591	Goossens *et al.* 1999 Marangoni *et al.* 2005a
Qualicode *B. burgdorferi* WB (Immunetics)	*B. burgdorferi* proteins	Marangoni *et al.* 2005b
B. burgdorferi WB (MRL)	Total sonicate of *B. garinii*	Goossens *et al.* 1999

The MarDX *B. burgdorferi* Marblot strip test system, the BBI Lyme WB test kit and the Viramed *Borrelia* Virablot test kit were evaluated in the U.S. using borreliosis positive and negative sera. The sensitivity and specificity of the different test kits

were evaluated. The results of this research are shown in Table 7. Because of the high specificities of the Marblot and the BBI Western blot, these tests may be more appropriate as a second step in two-tiered diagnosis (Mogilyansky *et al.* 2004).

Table 7 Sensitivity, specificity, and overall agreement of three Western blot tests (Mogilyansky *et al.* 2004)

IgG	Sensitivity (%)	Specificity (%)	Overall agreement (%)
Marblot	85	97	91
Virablot	85	90	88
BBI WB	81	97	89
IgM	Sensitivity (%)	Specificity (%)	Overall agreement (%)
Marblot	62	97	81
Virablot	85	77	81
BBI WB	77	100	89

The sensitivity and specificity of the Euroimmun Euroline-WB and Immunetics Qualicode *B. burgdorferi* WB were compared using sera from Italian patients with culture-confirmed first stage Lyme disease and potentially cross-reacting sera. Both of the IgM tests were 100% specific. The Euroline-WB against IgM was 13.3% sensitive, while the Qualicode *B. burgdorferi* WB was 8.3% sensitive. There were larger differences between the IgG tests. For the Euroline-WB the sensitivity was 68.3% and the specificity was 80.0%. The sensitivity of the Qualicode *B. burgdorferi* IgG WB was 26.7% and scored a specificity of 100% (Marangoni *et al.* 2005b).

In a comparison between the Virotech EcoBlot *Borrelia* and the Mikrogen recomBlot the results were very similar. Because of the higher sensitivity, the use of a of a multispecies Western blot was advised (Marangoni 2005a).

A comparison between the commercially available MRL and Genzyme Virotech IgM/IgG Western blots was made. The sensitivity and specificity were evaluated using sera from Lyme borreliosis patients, healthy control subjects and patients with diseases other than Lyme disease that could induce cross-reactivity. The results are shown in Table 8 (Goossens *et al.* 1999). The differences between those result are not remarkable, however the 4% sensitivity of the MRL IgG Western blot in early Lyme disease would invalidate the results of the EIA. The use of the Genzyme Virotech WB is therefore preferable.

Table 8 Sensitivity and specificity (%) of two WBs (Goossens *et al.* 1999)

IgM test	Percent sensitivity		Percent specificity	
	ELB	LLB	Healthy controls	Non-Lyme patient controls
MRL WB	46	54	98	90
Genzyme Virotech WB	50	62	89	80
IgG test	Percent sensitivity		Percent specificity	
	ELB	LLB	Healthy controls	Non-Lyme patient controls
MRL WB	4	46	97	100
Genzyme Virotech WB	27	46	82	91

ELB, early Lyme borreliosis; LLB, late Lyme borreliosis

Performances of the Mikrogen recomBlot *Borrelia* and the Eurimmun Euroline-Western blot were evaluated using sera from patients with various manifestations of Lyme borreliosis. The Euroline-Western blot IgG had the highest sensitivity, although the sensitivity of the Mikrogen recomBlot IgM was higher than that of the Euroline-WB IgM. When IgM and IgG results were combined, the sensitivity of the Western blot test kits were similar (Matthys *et al.* 2007).

These data do not indicate that no immunoblot is distinctly preferable. Differences between the performances of Western blot assays appear to be limited.

Polymerase Chain Reaction

PCR could form an alternative diagnostic tool for diagnosis of LB. Targeting the borrelial DNA, this technique is able to show the presence of the spirochete directly. Although this method is generally highly specific, insufficient sensitivities could hamper clinical application (Dumler 2001). There are different types of PCR assays which may be attributed for clinical diagnosis. The conventional and nested PCR methods give qualitative results, while competitive and real-time fluorescence PCR yields quantitative results (Aguero-Rosenfeld *et al.* 2005). Advantages associated with the use of real-time PCR is that this technique is able to discriminate between different *Borrelia* species (Rauter *et al.* 2002). Moreover, the real-time PCR procedure is much faster than conventional PCR methods (Espy *et al.* 2006), which would improve its suitability for routine use. The performances of PCR methods for the diagnosis of Lyme disease are influenced by many factors, including the size of the amplicon, detection method, extraction procedure, possible inhibition of the PCR by substances in the test sample and the selected target DNA (Schmidt 1997; Joss *et al.* 2008). The genetic material that is targeted for diagnosis may be plasmid-borne genes, chromosomal genes or gene segments of the 16S rRNA or 5S/23S rRNA gene intergenic spacer region (Wilske *et al.* 2007). Performances of PCR assays also differ with the sample material, depending on the concentration of spirochetes in the specimen (Schmidt 1997). The appropriateness of PCR diagnosis using patient blood, skin specimens, cerebrospinal fluid, synovial fluid and urine therefore need to be evaluated independently.

Blood, serum and plasma samples
The results of researches on the use of PCR in blood and serum samples are very diverse. A number of studies show that the sensitivity of this procedure is sufficient for reliable diagnosis of Lyme disease. Real-time PCR using primers that target the *flagellin* gene conducted in blood samples which were spiked with *Borrelia* indicated that this method is able to detect the spirochete's presence (Joss *et al.* 2007). 16S rRNA targeting PCR assays also seemed reliable for the examination of serum samples. 100% of the serum samples from patients with Lyme borreliosis was identified as positive, along with 40% of the borderline patient samples, while *Borrelia* was detected in none of the serum-negative samples (Santino *et al.* 2008). Another study that used nested PCR targeting the *rrs* gene found a 73.3% sensitivity of PCR in blood samples of Lyme patients with erythema migrans at the moment of diagnosis and a sensitivity of 52.3% after four weeks of antibiotic therapy (Kondrusik *et al.* 2007). Different studies, on the other hand, discourage the use of PCR in blood and plasma for routine diagnosis of Lyme disease. One study that used primers against the *B. burgdorferi* RNA polymerase C gene found sensitivities ranging from 0% to 40% in blood samples from patients with Lyme disease, depending on the severity of the symptoms (Goodman *et al.* 1995). The sensitivities in blood samples of patients with neuroborreliosis were 10.4% for both nested PCR targeting the intergenic *rrf-rrl* region and the plasmid encoded *OspA* gene (Cerar *et al.* 2008b). Using nested PCR against the *fla* gene, a sensitivity of 11.1% was found in blood samples from patients with Lyme borreliosis (Chmielewska-Badora *et al.* 2006). A different study was able to detect *Borrelia* in only 6% of the plasma samples from LB patients by using real-time PCR targeting the *flaB* gene (Coulter *et al.* 2005). Because the results of studies on the reliability of PCR in blood, serum or plasma samples are contradictory, it would be unwise to rely on this method as a routine procedure for diagnosis of LB.

Skin biopsies
The use of PCR has proven to be a considerably reliable method for the detection of *B. burgdorferi* in skin biopsies. The sensitivities of a nested PCR assay targeting the intergenic *rrf-rrl* region and a PCR targeting the *flagellin* gene tested in skin specimens of patients with erythema migrans were 71.3% and 24% respectively (Cerar *et al.* 2008a). Nested PCR targeted against chromosomal sequences had a sensitivity of 69% in biopsies from erythema migrans-lesions and a sensitivity of 61% in acrodermatitis chronica atrophicans-biopsies (von Stedingk *et al.* 1995). For EM-samples, a sensitivity of 75% was found for both PCR targeting the *OspA* gene and the 5S-23S intergenic region. For ACA the sensitivities were 62.5% and 41.67%, respectively (Rijpkema *et al.* 1997). One study showed that the 64% sensitivity of nested PCR targeting the *flaB* gene is lower than the 80% sensitivity of quantitative PCR targeting the *recA* gene (Liveris *et al.* 2002). Similar results were obtained in a different study which found 80.9% sensitivity with quantitative PCR against the *recA* gene in EM-biopsies and a sensitivity of 63.8% for nested PCR targeting the *flaB* gene (Nowakowski *et al.* 2001). It has been shown that PCR remains a reliable method if samples of freshly frozen EM and ACA biopsies are used. The sensitivity using primer sets that target 23S rRNA was 73% and when primers against 66-kDa protein were used the sensitivity of PCR in frozen tissues was 79%. PCR performed on paraffin-embedded tissue samples showed reduced sensitivity, namely 44% and 52% respectively (Brettschneider *et al.* 1998). Also, even though 16S rRNA based PCR in skin biopsies of patients with erythema migrans yielded 71% sensitivity, this method was only 17% sensitive in skin biopsies of patients with neuroborreliosis. This indicates that PCR in skin samples is not a suitable method to diagnose Lyme neuroborreliosis (Lebech *et al.* 2000). However, the fact that PCR in skin biopsies is relatively reliable for EM-samples and ACA-samples implies that this procedure is useful in both early and late stages of Lyme borreliosis with cutaneous manifestations (von Stedingk *et al.* 1995).

Synovial fluid and membrane specimens
PCR assays targeting the plasmid encoded *OspA* gene and the 16S rRNA proved able to readily detect *B. burgdorferi* in synovial fluid samples from patients with Lyme arthritis. The *OspA* targeting methods yielded an average sensitivity of 68.18%, while the 16S rRNA targeted PCR was 47.73% sensitive. No borrelial DNA was found in any of the control samples for either PCR assay (Nocton *et al.* 1994). Another study found a sensitivity of 71% for PCR targeting the *OspA* gene in synovial fluid of patients with untreated Lyme arthritis (Frossard *et al.* 1999). Sensitivities of 17.9% and 20% have been found for f*lagellin*-based real-time PCR in synovial fluid and synovial membrane specimens, respectively (Schwaiger *et al.* 2001). The data implicate that PCR in synovial fluid could be a valuable supplemental tool in diagnosis of Lyme borreliosis for patients with rheumatological manifestations.

Cerebrospinal fluid
The average sensitivity of PCR assays in cerebrospinal fluid (CSF) is very low. *Flagellin*-based PCR had a sensitivity of only 1.8% in the CSF of patients with probable neuroborreliosis (Schwaiger *et al.* 2001). A sensitivity of 20.6% was found when the CSF of patients with Lyme neurobrreliosis was examined using *OspA*-based PCR (Lebech *et al.* 1998). Sensitivities of 12.2% were found for nested PCR targeting either the *rrl-rrf* intergenic region or the *OspA* gene in the CSF of patients with neuroborreliosis (Cerar *et al.* 2008b). In CSF samples from children with Lyme meningitis, a sensitivity of only 5% was found (Avery *et al.* 2005). Overall, PCR in cerebrospinal fluid does not seem suitable for LNB diagnosis. It has been suggested

that low sensitivities are due to the limited number of bacteria that are present in the CSF (Tang *et al*. 1999).

Urine
The use of urine samples for the diagnosis of Lyme disease has long been controversial, as the quantity of spirochetes in urine was undetermined and the urine may have an inhibitory effect on PCR assays. Some studies indicate that urine samples could indeed be used for diagnostic purposes. The sensitivity of nested *flagellin*-based PCR, for example, was found to be 79% for EM positive patients and 63% for patients with ACA (Aberer *et al.* 2007). According to different studies, however, the sensitivities of PCR in urine samples insufficient. 27% sensitivity was found for PCR targeting the 23S rRNA and 0% sensitivity for 66-kDA protein-based assays (Brettschneider *et al.* 1998). The sensitivities of 16S rRNA-based PCR in urine of patients with EM and neuroborreliosis were 13% and 7% respectively (Lebech *et al.* 2000). Another experiment found 0% sensitivity for nested PCR targeting the *rrs* gene in urine of Lyme patients at the moment of diagnosis or after 4 weeks of antibiotic therapy (Kondrusik *et al.* 2007). Urine samples from patients with EM that were examined by nested PCR with primers targeting *OspA* followed by real-time PCR detected *Borrelia* DNA in 8.33% of the samples. When the experiment was repeated targeting the *flagellin* gene no positive results were found. Examination of urine from healthy donors spiked with *Borrelia* or *Borrelia* DNA indicated that the amount of DNA in the urine may be too low for detection (Rauter *et al.* 2005). The evidence suggests that the use of urine samples is not suitable for PCR analysis of LB.

In conclusion, it seems that PCR can only contribute to diagnosis of Lyme disease when it is used to examine skin specimens of erythema migrans or acrodermatitis chronica atrophicans and to test synovial fluid or membrane specimens for Lyme arthritis. The sensitivity of PCR in blood samples, CSF samples and urine samples is insufficient for diagnostic purposes. As large differences have been found between PCR assays within a specific sample, the choice of target DNA and other features of the PCR should be chosen carefully to achieve optimal sensitivity of the procedure.

CSF analysis in Lyme neuroborreliosis

Borrelia induced inflammation of the central and peripheral nervous systems leads to the neurological degeneration (Barsan *et al.* 2009), which may clinically be expressed as mononeuritis, radiculoneuritis or meningitis (Barsan *et al.* 2009). For the diagnosis of possible Lyme neuroborreliosis it has been suggested that at least two of the following criteria should be fulfilled: neurological symptoms, CSF pleocytosis and intrathecally produced antibodies against *Borrelia* (Mygland *et al.* 2010). All three characteristics should be present for diagnosis of definite LNB (Mygland *et al.* 2010).

Antibody indices
In case of Lyme neuroborreliosis, antibodies against *B. burgdorferi* are produced intrathecally (Mygland *et al.* 2010). To determine whether the source of antibody production lies inside the CNS, the amount of antibodies in the CSF and the concentration of serum antibodies are serologically established. The ratio of *Borrelia*-specific antibodies in the CSF to serum, referred to as the antibody index (AI), is elevated in patients with Lyme neuroborreliosis (Kaiser & Lücking 1993). There are several procedures to calculate the antibody index. First of all, the CSF to serum ratio of *Borrelia*-specific antibody titers (Blanc *et al.* 2007) or optical densities (ODs) of specific antibodies can be determined (Kaiser & Lücking 1993). These ratios are then divided by the CSF to serum ratio of total IgG (Blanc *et al.* 2007) and incidentally also IgM (Cerar *et al.* published ahead of print) or albumin (Klapper *et al.* 1981) to determine the antibody index. Alternatively, the same amounts of total Igs can be collected from the patient's CSF and serum to assess the ratio of specific antibodies present in each sample (Tugwell *et al.* 1997). It has been shown that these calculation methods are almost equivalent in their ability to demonstrate intrathecal antibody synthesis (Kaiser & Lücking 1993). Yet another calculation method does not account for the amount of total unspecific antibodies, but merely divides the OD of specific antibodies in the CSF by the serum OD and multiplies the outcome with the OD of the CSF minus the OD of the serum (Skarpaas *et al.* 2007, Bennet *et al.* 2008).

For antibody indices calculated based on the amount of total Igs, the AI that is considered to be significantly positive varies from ≥1.4 (Cerar *et al.* published ahead of print) to ≥2 (Kaiser & Lücking 1993). Antibody indices of patients with LNB vary between 0.9 and 98.7 with an average value of 13.8 (Kaiser & Lücking 1993). Approximately 3.5% of the antibody indices of patients with LNB lies between 1.4 and 2 (Kaiser & Lücking 1993). Variations in the positive AI threshold that is administered influences the sensitivity and specificity of antibody index assays. The threshold that will yield the most accurate results depends on the a priori chance of the test population. Moreover, a wide intermediate range will increase test accuracy, but reduce the number of cases in which antibody index determination can contribute to diagnosis of Lyme neuroborreliosis. The same holds for antibody indices calculated without correcting for the total amount of antibodies. A positivity threshold of ≥0.25 or ≥0.3 is generally applied with this calculation method (Skarpaas *et al.* 2007, Ljøstad *et al.* 2007, Bennet *et al.* 2008). As the sensitivities of AI determinations are furthermore affected by the impediments of serological testing a deliberate choice of utilized antigens is required.

Various studies have found sensitivities of antibody indices ranging from 56% to 80% in patients with definite Lyme neuroborreliosis (Blanc *et al.* 2007, Ljøstad *et al.* 2007, Roux *et al.* 2007, Cerar *et al.* published ahead of print). A negative AI in

patients with definite LNB has shown to correlate with reduced symptom duration (Ljøstad *et al.* 2007). One study, however, also found positive antibody indices in 18.8% of patients with TBE infection (Cerar *et al.* published ahead of print). Yet a different study that tested the specificity in patients with a range of other disease found 98% specificity for the use AI assays in LNB diagnosis (Blanc *et al.* 2007).

In the U.S., antibody indices are not commonly used to identify Lyme neuroborreliosis. It has been shown that the use of AIs has lower sensitivities in American than in European LNB patients, which may be due to the occurrence of different *Borrelia* species (Steere *et al.* 1990). In Europe, most of the patients with Lyme neuroborreliosis are infected with *Borrelia garinii*, though this pathogen is absent on the American continent (Mygland *et al.* 2010). The sensitivity of antibody index assays is sufficient for clinical use for diagnosis in European patients with manifestations that indicate neuroborreliosis.

Pleocytosis
Monocytic or lymphocytic pleocytosis in the CSF indicates the presence of a neurological inflammation (Pachner & Steiner 2007). Analysis of CSF obtained by lumbar punctures have shown elevated numbers of B, T and plasma cells, as well as higher numbers of monocytes and natural killer cells (Holub *et al.* 2002, Cepok *et al.* 2003). It has been shown that 68% of children with facial nerve palsy has elevated white blood cell count, protein level, or both in the CSF (Belman *et al.* 1997). The finding of CSF abnormalities therefore implicates *B. burgdorferi* infection of the central nervous system. However, the possible absence of pleocytosis in the early stages of Lyme disease may reduce its diagnostic value in acute LNB (Stanek *et al.* published ahead of print). Moreover, because recruitment of lymphocytes into the central nervous system is not specific for Lyme neuroborreliosis (Holub *et al.* 2002, Halperin 2010), demonstration of pleocytosis is only suitable as a complementary test method. Identification of cytokines in the CSF may form an alternative, though equally unspecific, method to identify the inflammatory response in the nervous system (Kondrusik *et al.* 2004).

Because of the *Borrelia* specific character of antibody index determination, this method is preferable over the demonstration of white blood cell activation. However, due to the suboptimal sensitivity of serological testing clinical manifestations continue to play a dominant role in diagnosis of Lyme neuroborreliosis.

Discussion

The Quick ELISA C6 antibody assay, which is based on a synthetically produced peptide that mimics part of the VlsE protein of *Borrelia burgdorferi*, appears to be the most appropriate immunoassay for Lyme diagnosis among the enzyme immunoassays evaluated here. The IR6 of the VlsE protein is expressed early after infection and conserved among *Borrelia burgdorferi* genospecies (Liang *et al.* 1999). Although the Quick ELISA C6 assay has been put forward as an alternative for two-tiered testing (Wormser *et al.* 2008), omission of a confirmatory test would be imprudent due to the suboptimal performance of the immunoassay.

The differences between commercially available second-tier Western blotting assays seem to be limited. There is no large difference between the validity of blotting assays based on recombinant antigens, native antigens, or a combination of both. However, no analysis that is specifically aimed at the comparison of different immunoblots for Lyme diagnosis has been performed to date.

The sensitivity of PCR in blood, cerebrospinal fluid and urine is insufficient to render a substantial contribution to diagnosis of Lyme disease. It can however be a valuable tool to detect the presence of *Borrelia* in synovial specimens which elicits Lyme arthritis. PCR may also be used in skin biopsies from patients with erythema migrans or acrodermatitis chronica atrophicans. Yet the presence of these symptoms provides a rather evident indication of Lyme borreliosis, so the added value of PCR in skin biopsies from patients with clinical manifestation of LB is only moderate.

In case of clinical manifestations that indicate Lyme neuroborreliosis, antibody indices are deduced from the CSF to serum ratio of *Borrelia*-specific Igs. LNB can be established based on a positive antibody index, which demonstrates intrathecal production of antibodies. Pleocytosis forms an additional indication, but as the occurrence of a neurological inflammation is not specific for LNB it should not solely be relied on.

Medical practitioners should be well aware of the fact that due to the lack of a gold standard in laboratory methods, the results are not absolute and should therefore not be bluntly relied on. Assessment of a number of factors is required to obtain insight into the situation of a potential Lyme patient. It is therefore important that diagnosis is based on a combination of clinical symptoms, laboratory testing and knowledge of the incidence of Lyme disease in a population. Differences between the occurrence of Lyme borreliosis in Europe and North America with respect to the identity of the pathogen have already been mentioned. In the Netherlands 10-30% of the ticks is infected with *Borrelia burgdorferi* (Anonymous 2005). Approximately 17,000 patients visited a general practitioner exhibiting erythema migrans in 2005, though reflecting on the commonness of asymptomatic Lyme disease it can be assumed that the actual number of Lyme patients in this country is markedly higher (Anonymous 2005). The relatively high a priori chance in the Netherlands should be considered during evaluation of clinical indications and laboratory data for diagnosis of Lyme borreliosis.

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