Journal of Bacteriology and Parasitology

ISSN: 2155-9597

Gruber. J Bacteriol Parasitol 2013, S:5
DOI: 10.4172/2155-9597.S5-004

Review article Open Access

Multiplex Bead Based Immunoassays For The Serodiagnosis of Lyme Borreliosis

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Abstract

Laboratory diagnosis of Lyme borreliosis is based on the detection of specific IgG and IgM antibodies against relevant antigens of *Borrelia burgdorferi*. Serological diagnosis usually is performed by a two-step procedure. Where immunoassays for antibody screening exhibit high sensitivity, second-line tests, e.g. Western-blot or line-blot assays,show high specificity, and are used for confirmation of positive screening results. In addition to serology, in certain cases *Borrelia spp*. can be detected directly, for instance in synovial fluid from patients with Lyme arthritis by PCR or bacterial culture. With the development of multiplex technology, e.g. bead-based immunoassays, multiple antibodies to distinct bacterial antigens can be detected in a single run. Detection systems used in this context, e.g. analysers based on flow cytometry, can be highly standardised and automated. Furthermore, these analysers can be connected bidirectionally to an order-entry based laboratory information system, with random access for high throughput analysis. Thus, multiplex bead assays may have the power to replace the current two-step procedure.

Keywords: Neuroborreliosis; Cerebrospinal fluid; Erythema chronicum migrans; Immunoblot; Lineblot

Introduction

In Europe, there are two common infectious diseases transmitted by ticks. Infection by FSME-virus leads to meningoencephalitis, while Lyme borreliosis is caused by the spriochetal bacterium, *Borrelia burgdorferi*. Other tick-borne diseases, e.g. relapsing fever caused by other *Borrelia* species (*B. hermsii, B. duttoni,* etc.), spotted fever (*Ricketsia ricketssii, R. coronii*, etc.), Ehrlichiosis (*Anaplasma phagocytophilium*, formerly named *Ehrlichia chaffeeensis*), or Babesiosis (*B. microti, B. divergens*), are rare [1]. In the USA, almost only infection with *B. burgdorferi* sensu stricto results in development of Lyme borreliosis, while in Europe, borreliosis is commonly caused by infection with *B. afzelii* and *B. garinii*. Infections with *B. spielmanii* and *B. bavariensis* are rarely found in Europe, and in contrast to the USA, also of infections with *B. burgdorferi* sensu stricto. *B. burgdorferi* sensu stricto is mainly associated with arthritis (USA and Europe), while erythema migrans is most frequently caused by *B. afzelii,* and *B. garinii* most frequently causes neurological disorders [2].

Manifestations of Lyme disease are often classified into three stages, according to corresponding incubation periods from infection to clinically overt manifestation (Table 1). There is no strict discrimination of the stages and late manifestations may occur without early disease symptoms. Consequently, the updated European case definition does not imply this classification [2]. The main early manifestation is erythema chronicum migrans. This characteristic skin disease occurs 3 to 30 days after tick-bite. Benign lymphocytoma may occur within a few weeks after tick-bite. Some weeks to months after tick-bite, there is a hematogenous dissemination of the pathogens. Although various organs can become infected, neuronal symptoms are the prominent feature of systemic infection, for instance, meningitis, radiculitis, paralysis of cerebral nerves, especially facial palsy or rarely carditis. Late stage disease occurs months to years after primary infection. Especially in USA, arthritis (Lyme arthritis) is a common symptom of late stage borreliosis, but also encephalomyelitis and acrodermatitis chronica athrophicans may occur. *B. burgdorferi* infections can effectively be treated with antibiotics, but a few patients may have some long term sequelae, especially if the diagnosis is delayed. Currently, no *Borrelia* vaccine for human use is available [3].

	Stage 1	Stage 2	Stage 3
	Local infection	Early disseminated disease	Chronic infection
Incubation time	3 to 30 days	weeks to months	months to years
Diseases	Erythema chronicum migrans	- benign lymphocytoma - meningitis* - radiculitis* - cranial nerve paresis* - cardiditis	- Lyme-arthritis - encephalomyeliti - acrodermatitis chronica atrophicans
Serology IgM-positive	20-90%	10- 40%	0- 30%
Serology IgG-positive	up to 50%	up to 90% (depends on disease duration)	almost 100%

* Antibody tests in CSF and antibody index should be performed in suspected neuroborreliosis

Table 1: Stages of Lyme disease.

Laboratory Diagnosis

Serological laboratory diagnosis of Lyme disease is usually done in a two-step procedure, beginning with a screening assay to detect IgG and IgM *anti-borrelia* antibodies. Screening assays should be highly sensitive to avoid missing *Borrelia*-infected patients. However, high sensitivity usually results in low specificity of laboratory test systems, especially for IgM antibody assays. Moreover, unspecific reactions are common in almost all assays for detection of IgM antibodies. Therefore, it is essential to confirm reactivity observed in screening assays by highly specific second-line test systems.

In screening assays to detect anti-Borrelia antibodies, whole cell lysates, a mixture of purified native or recombinant antigens, or

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Received November 19, 2012; **Accepted** February 04, 2013; **Published** February 09, 2013

Citation: Gruber R (2013) Multiplex Bead Based Immunoassays For The Serodiagnosis of Lyme Borreliosis. J Bacteriol Parasitol S5-004. doi:10.4172/2155-9597.S5-004

a combination of these are used. These antigen preparations have to fulfil sophisticated demands. On the one hand, antigens should cover a broad diversity of relevant antigens of the different pathogenic *Borrelia* species. On the other hand, antibodies targeting non-pathogenic *Borrelia* species or other bacteria, e.g. *Treponema pallidum*, should not bind to the antigen preparations. Second-line confirmatory tests often are Western-blot or line-blot. Initially, crude native homogenates of whole bacteria were used in screening and second-line tests. More recently, purified native or recombinant single antigens have been used for construction of test assays. However, the two-assay principle is currently being challenged, since recombinant proteins and peptides combine high specificity, while not loosing sensitivity [4,5].

Multiplex immunoassays are appropriate tools to analyse complex antibody response patterns. In parallel to the development of DNA-CHIPS, antigen-CHIPS have also been designed. Benefits result from the possibility to examine a large number of antibody reactions, while using a minimum amount of the patient's serum. This is especially relevant for paediatricians, but also for veterinarians. A major drawback of classic CHIP-formats was the difficulty to standardize quantitative CHIP assays, and thus, generate reproducible results on glass slides containing spotted antigen preparations. In contrast, it is easier to standardize other multiplex formats generating quantitative results, for instance bead assays. However, very expensive analysers such as flow cytometers are necessary to evaluate antibody reactions. Fortunately, bench-top analyzers like the Luminex flow cytometer and well standardized commercially available test kits have been developed, allowing the use of multiplex assays for routine diagnostics in the medical laboratory [6].

Multiplex assays based on bead technology

The commercially available bead assays are distributed in Germany by MIKROGEN, Neuried (recomBead and Multimetrix *Borrelia* IgG/IgM); PROGEN, Heidelberg, (Multimetrix *Borrelia* IgG/IgM Test) and Virion/Serion, Würzburg (Multianalyt *Borrelia* IgG/IgM). All of these companies distribute second-line tests and Multimetrix, also a screening test for IgM and IgG. Antigens used in this screening assay for IgM antibody detection is a lysate from *B. afzelii,* recombinant OspC from *B. afzelii,* and recombinant OspC from *B. garinii*, while for the IgG test, only lysates from *B. garinii* and *B. afzelii* are used. Two second-line tests use *Borrelia* lysate, while one assay (recomBead) omits lysate [6,7]. Table 2 shows some of the antigens used in the bead assays in comparison to some blot tests. For some antigens, for instance p83/100, p39, OspC, p41 (flagellin) internal fragment, Osp17 and p58 improvement of test performance has been documented in these multi-antigen tests. For other antigens, the selection criteria and the specificity of individual antigens in control populations often are not well documented [5,7,8]

Borrelia specific antigens used in immunoassays

In Europe, primarily three species of the *Borrelia* genogroup, namely *B. afzelii, B. garinii* and *B. burgdorferi* sensu stricto, but also *B. spielmanii* and *B. bavariensis* are pathogenic for humans. These species exhibit proteins which are strongly conserved with high homology, but also proteins showing inter-species variability. To cover a maximum of relevant immune reactions, careful selection of antigens is essential. Ideally, antigens showing high homology between various *Borrelia* species should be used.

Consequently, usage of those antigens will allow detection of antibodies even when species causing infection differ from that providing antigens for test assay. A further aspect to consider in antigen selection is the fact that antibody production to certain antigens changes during the course of infection. Therefore, in addition to the presence of IgG and/or IgM antibodies, the reactivity against such antigens can help to differentiate between early and late infection. The determination of antibody avidity would be an additional way to assess the temporal course of *Borrelia* infection; however, to date no commercial assays are available to analyse antibody avidity of *anti-borrelia* antibodies.

Some antigens of other bacteria-for instance flagella proteins of spirochetes and *Enterobactericeae*-show structural similarities to the corresponding *Borrelia* antigens. Therefore, usage of these epitopes could decrease specificity of test assays due to binding of cross-reacting antibodies. According to European recommendations [5], a clear positive reaction of at least two specific antigens in an immune blot is considered sufficient to interpret the assay result as "positive".

In addition to the differentiation of immune reactions against early and late antigens, and the presence of IgG and/or IgM antibodies, an increasing number of positive reactions is expected during an ongoing infection. While OspC and VlsE are the most sensitive antigens for an IgM or IgG response, respectively, most second-line assays, including the multiplex bead assays, use at least five and sometimes more than 10 different specific antigens [9-11].

In table 3, some relevant *Borrelia* antigens are listed. As already discussed, the use of recombinant or purified native antigens is preferable to whole cell lysates or classical Western-Blot assays, using electrophoretically separated antigens of whole cell lysates. Therefore, line-Blots and multiplex assays with recombinant antigens are now most commonly used in routine diagnosis. Another important improvement in the diagnosis concerning sensitivity and specificity of the immuno assays was the introduction of *in vivo*-induced antigens which are not expressed in cultured bacteria, but only in the infected host, and which are therefore, missing in whole cell lysates. The first such antigen to be introduced and still the most prominent is VlsE (vmp-variable major protein-like sequence expressed), which is now complemented with Dbp(A) (Decorin binding protein A) in some routine assays [10,12,13].

Interpretation of serological results

Guidelines from microbiological societies are available to

	Antigen	VlsE	p100	p83	p58	p43	p41	p39	p30	OspA	OspC (p22)	p21	p19	Osp 17 Dbp(A)	p14	lysate
Line-Blot	EUROLINE IgG	x		x	x		x	x			x	x	x	x		
	EUROLINE IgM	x			x		x				x					
	Viramed IgG	x		x	x	x	x	x	x		x	x		x	x	
	Viramed IgM	x					x	x			x			x		
Bead	recomBead IgG / IgM	x	x		x			x		x	x			x		
	Multianalyt IgG/IgM	x	x		x		x				x			x		x
	Multimetrix IgG	x	x		x		x	x		x	x			x		x
	Multimetrix IgM	x	x				x	x		x	x			x		x

Table 2: Antigens used by different manufacturers in Bead- and Line-Blot-Assays (selection).

Molecular weight in kD	Antigen	Meaning for diagnosis	Antibody reactions
p100/p83	(p83 is degradation product of p100)	+++	Typically positive in late stages, but early antibody reaction possible
p58		+	Often in stage 3
p43/p45		+	Mostly in stage 3
p41	Flagellin	+	Very early reactive, cross reactions with some antigen preparations
p39	BmpA (Borrelial membrane protein A)	+++	Rather specific for late IgG-response; sometimes also early
p31	OspA (Outer surface protein A)	++	Late response
p30		+	Not clearly defined
p22/p25	OspC	+++	Very early seen; mostly the first specific reaction of the early IgM response; species specific, especially the IgG response
p21		++	Not clearly defined
p19	OspE	+	Not clearly defined
p17/p18	Dbp (A) Decorin binding protein A	++	Surface protein, species specific response
p14	internal protein	+	Mostly in early infection
VlsE	VlsE=vmp (variable major protein) like sequence expressed, surface lipoprotein	++++	IgG in early and late infection, intra thecal response in neuroborreliosis

Table 3: Antibody response to specific antigens used in immuoassays.

facilitate interpretation of Western- or line-blots results. However, recommendations are not available for interpretation of bead assay results [5]. Comparing these guidelines to the recommendations of the manufacturers of bead assays, it appears that manufacturers have adopted some recommendations for interpretation of test results generated when using bead assays. As the bead assays give quantitative results, the antibody titer is incorporated into the interpretation guidelines for some assays. Furthermore, the diagnostic relevance of certain antigens is used for classification. For example, according to the Progen protocol, one clear positive or two weak reactions should be interpreted as "Lyme borreliosis not reliably excluded". If more than one antigen shows a strong positive reaction, the overall interpretation is "strong suspicion of Lyme borreliosis". Another way to interpret results as "negative", "borderline" or "positive", respectively, is suggested for other assays.

Typical serological constellations are shown in table 4. The diagnosis of different disease stages according to serological results is only possible if the anamnestic and clinical situations are known. Even in this case, many results are not conclusive. Sometimes, the follow up of clinical signs but also serological results, can help to obtain a final diagnosis [1,3].

Tick bite: There is no indication to perform serological diagnostics following a tick bite, when the patient lacks symptoms of borreliosis. The serological response early in infection (acute invasion) will be negative, because formation of IgM antibodies usually starts only two weeks after *Borrelia* infection. There is no recommendation for antibiotic therapy, if seroconversion is noticed by chance when symptoms are lacking. More than 95% of infections are clinically inapparent [14].

Early local disease: In this stage of the disease, serological examination usually is not necessary if a typical erythema chronicum migrans is present. Erythema occurring within the first two days after infection probably consists of unspecific immunological reactions. However, in case of dubious symptoms, serological analysis is indicated. With up-to-date immunoassays, IgM antibodies may be detected in 50-90% and IgG in 10-50% of patients.

Early disseminated disease: Manifestations of early disseminated *Borrelia* infections are neuroborrelosis, accompanied by meningitis and/or neural paralysis, e.g. facial palsy. In these cases, serological tests are very useful, and if there is clinical suspicion of *neuroborreliosis,* analysis of antibody titres in serum and cerebrospinal fluid should be performed to assess intrathecal production of antibodies targeting *Borrelia*. In up to 90% of neuroborreliosis patients, intrathecal IgG production is present, and intrathecal IgM production is seen in 15-70% of neuroborreliosis cases.

Neuroborreliosis is proven if characteristic symptoms are present, and also pleocytosis in cerebrospinal fluid combined with intrathecal production of anti-*Borrelia* antibodies [15].

Late disease manifestations: Patients suffering from late stage diseases are always positive for IgG antibodies. Characteristically in the confirmatory assay, antibodies to many antigens are present. An active late stage disease with negative serology is an absolute rarity, or even an incorrect diagnosis. A positive IgM-result with negative IgG does not support the diagnosis of a stage 2 or 3 disease, but is rather a coincidence of early infection, a persistent IgM-Titer, or an unspecific IgM-reaction, together with an other disease mimicking borreliosis [1,3].

Critical points to consider in the interpretation serological test results

A negative serological result does not exclude an early infection. In unclear clinical situations, a follow up should be done in 3-6 weeks. If early antibiotic therapy has been initiated, antibody production may be blocked, e.g. no antibodies are induced at all or the switch of IgM to IgG antibody production does not occur, resulting in the persistence of IgM and lack of IgG antibodies for months, or even for years. Moreover, in some patients, no IgM is induced, even if later on in the course of infection specific IgG antibodies can be found. IgG usually persists over years or lifelong also in inapparent infections, and sometimes even with high titers with many positive bands in an immunoblot. Unspecific neurological symptoms, for instance, fatigue and depression, but also arthralgia often coincide with IgG antibody finding, especially in endemic regions, or in persons with high risk for tick bites, e.g. forest workers. Unspecific antibody binding, especially in screening assays, might occur as a consequence of infections caused by other microorganisms, e.g. EBV, *Treponema palldium*, but also as a consequence of autoimmune diseases [5,16].

Discussion

The basis of laboratory diagnosis of Lyme borreliosis is the detection of specific IgG and IgM antibodies against relevant antigens of pathogenic *B. burgdorferi* strains. Serological diagnosis is based on a two-step approach, starting with a screening assay (e.g. ELISA), and if reactive, a second-line test. Usually this confirmation assay is a test simultaneously detecting various antibodies to specific bacterial antigens, e.g. a Western- or line-blot. This two assay test strategy is based

IgM		IgG		Interpretation
Screening	Confirmation	Screening	Confirmation	
neg	neg	neg	neg	No late Borreliosis; (CAVE: early infection with negative serology; depending on the clinical situation a control in 10-14 days should be recommended)
pos	neg	pos	neg	Unspecific reactions; the interpretation of the serological result has to be "negative" (CAVE: very early infection with positive screening test but negative confirmation test; depending on the clinical situation a control in 10-14 days should be recommended)
pos	pos	neg	neg	Early infection (CAVE persisting IgM from a former infection without IgG-production)
pos	pos	pos	pos	Early infection with IgG already positive (CAVE: former infection with persistent IgM)
neg	neg	pos	pos	Active infection stage 2 or 3 or persistent IgG after spontaneous healing or sucessfull treatment

Table 4: Interpretation of typical serological antibody constellations.

on the principle that the screening assay is a cheap and very sensitive high throughput method. Second-line confirmation tests should be as sensitive as screening assays, but specificity should be significantly higher. However, these tests are expensive and testing procedure is time consuming [5].

There are few data to support the assumptions of sensitivity and specificity of the combined screening and second-line assay. Using confirmation tests in a high frequency screening setting, it may turn out that second-line confirmation tests are less specific than assumed, because the pre-selection of the screening test which gives an immense increase of pre-test probability, is missing. Probably confirmation tests are also less sensitive because per definition, a positive screening assay is ignored and overruled by a negative confirmation assay, and as a consequence, the reactivity of the screening assay will be called "unspecific".

With the introduction of multiplex technology, e.g. bead based immunoassays, which can provide multiple results in a single run, the two-step procedure has to be reconsidered. There are some great potential advantages of the multiplex bead technology. This technology can be highly standardised and automated, and has the possibility to perform accurate cut-off calibrations and to produce quantitative results. The "read out" procedure is automated, as opposed to subjective visual reading or semiquantitative scanning of blot assays. Internal controls can be run in each sample, and other biomarkers may be added to the same assay. Furthermore, analysers like flow cytometers may be used bi-directionally, connected to an order-entry based laboratory information system, with random access for high throughput analysis. Therefore, this technique saves time and money in routine analysis because there is no necessity to wait for results of the second-line tests. As mentioned above, this technology allows inclusion of antigens from other tick-borne diseases, e.g. babesiosis or even the detection and quantitation of markers of inflammation, such as CXCL13. The concentration of this chemokine seems to increase very early in the cerebrospinal fluid of neuroborreliosis patients, before the production of specific antibodies. Furthermore, increase of CXCL 13 concentration seems to be highly sensitive in the case of *neuroborreliosis,* but the specificity may be as low as 63%. Up to now, there is not enough evidence to recommend CXCL13 test as a routine diagnostic tool, or in follow-up after treatment [7,15,17].

The possibility to obtain quantitative results when using multiplex assays alleviates calculations of antibody index, and therefore, improves determination of intrathecal antibody production [6,15].

Immunoblots and bead based assays are quite expensive. This may be a reason why in the US it is discussed to use, for example, a VlsE based ELISA alone. The modern ELISA assays are already quite effective. However, studies are necessary to evaluate the performance of these ELISAs, in comparison to bead based assays and the utility of multiple antigens. The use of more antigens may even decrease specificity, and thus, diagnostic accuracy highly depends on good scoring algorithms. In conclusion, the introduction of multiplex assays for routine serologic diagnosis of borreliosis has the power to replace the classical two-step approach, and as a consequence, also assay formats like ELISA and Western- or line-blot. However, detailed studies with well characterized patients suffering from different disease states to confirm sensitivity and specificity of the multiplex assays, are still necessary.

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