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Serodiagnosis of Lyme borreliosis with bead based immunoassays using multiplex technology

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ABSTRACT

The serological diagnosis of Lyme borreliosis is accomplished by the detection of IgG and IgM antibodies specific for relevant antigens of the spirochetal pathogen *Borrelia burgdorferi*. Instead of the usual enzyme immune assay for screening and the Western blot technique for confirmation, bead based multiplex assays with multiple simultaneously performed distinct reactions can provide quick, automatically derived and reliable results in a single run by flow cytometer technology. The broad analytical dynamic range of assay signals and the high sensitivity and specificity of the multiplex formats allow even for a reliable use in CSF based analyses for antibody specificity index in supposed neuroborreliosis. Fluorescence intensity of the bead based reactions can be transformed into quantified values as U/ml, either for each single antigen or summed up for a group of relevant key antigens. Additionally or alternatively distinct reactions of single bead populations can be transformed to Western blot band equivalents. Internal and external quality controls with the multiplex systems show characteristic data equivalent to the conventional assay formats, so that the advantages of the multiplex assays are ready for use in the routine diagnostic laboratory.

1. Serodiagnosis of Lyme borreliosis

Lyme borreliosis is caused by infection with the Gram negative spirochetal bacterium *Borrelia burgdorferi*. It is a silvatic anthropozoonosis with a natural reservoir of infectious agents in small rodents (esp. mice), birds and deer. Transmission to humans is usually accomplished by ticks of the species *Ixodes ricinus* and *Ixodes dammini* colonized with the bacterium [1]. While chronic inflammatory impairment of large joints (predominantly hip and knee) is the main problem in the United States, the infection can also cause acute and chronic disease of the skin, joints, central and peripheral nervous system, heart and other organs [2]. This is frequently seen in Northern and Western Europe and other parts of the world with moderate climatic conditions. The main reason for the different clinical manifestation of Borrelia infection in several geographical localisations was found by the detection of distinct pathogenic subspecies contributing to a *Borrelia burgdorferi* complex, namely *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, *Borrelia garinii* and *Borrelia spielmanii* in Europe [3]. The pathogenic role of *Borrelia valaisiana* for humans is of uncertain evidence. In the United States *B. b. sensu stricto* is the only pathogenic subspecies identified so far. While *B. b. sensu stricto* is mainly associated with rheumatologic manifestations (US and Europe), *B. afzelii* is the most frequent pathogen for the erythema migrans, a centrifugally growing exanthema of the skin occurring 1–3 weeks after the stitch of a colonized tick. *B. garinii* is the subspecies most frequently found in neurological disorders [4,5]. No Borrelia vaccine for human use is available at the moment [6].

Laboratory diagnosis of Lyme disease is usually based on the detection of Borrelia antibodies of the IgG and/or IgM class. Cost effectiveness and ease of use have encouraged a two-step algorithm in Borrelia serodiagnosis: it has proved a successful diagnostic strategy to start with a sensitive screening test being followed by a specific confirmatory assay for reactive screening results. EIA and Western blot are the most common means employed for this purpose, respectively [7–9]. The antigens used in these assays have to fulfill two almost contradictory demands: they have to cover the broad antigenic diversity of several relevant Borrelia subspecies and simultaneously have to exclude reactions with non-pathogenic Borrelia strains and other potentially cross reactive agents. Most demanding are Gram negative rods of the genus *Enterobacteriaceae* with their flagella which share major immunogenic parts of the Borrelia flagellum. These circumstances give way to the preferred use of recombinant antigens in the serodiagnosis of Lyme borreliosis – and the employment of multiplex assays for routine diagnostic use in the medical laboratory.

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2. Relevant antigens in early and late Lyme borreliosis

More than 100 different proteins have been identified in Borrelia species so far, with a high inter-species and inter-strain variability [10,11]. Many of them are shared within the genus and a significant number can be found in more or less related prokaryotic taxa, too. To facilitate a reliable serodiagnosis of Borrelia infections it is necessary to select antigens which meet several critical criteria: first, they have to induce a measurable immune response in the infected host, ideally in all phases of infection; second, they should be highly specific for *B. burgdorferi*; third, they should cover all, but exclusively the pathogenic subspecies of the B. b. complex and fourth, they should ideally allow for the discrimination of fresh/acute, chronic or past, active or healed infections. It is evident that not one single antigen can meet such a diversity of criteria at one time, so there is a need for a suitable selection of antigens in a sophisticated cocktail. A small number of relevant antigens have turned out sufficient to meet the described criteria at least approximately [12,13].

Following German and European guidelines for the serodiagnosis of *Borrelia burgdorferi* infection [14] reaction in a patient's serum with two specific antigens in an immune blot is considered sufficient to establish the diagnosis, revealing OspC and VlsE as the most sensitive antigens for an IgM or IgG response, respectively [11,15]. The observation of a broad antigenic spectrum on the blot and reactions with antigens usually seen late in the host immune response can help to identify infections which have persisted for a longer time or possibly have evolved chronic. An overview upon the most relevant Borrelia key antigens is given in Table 1.

Different assay formats have turned out useful for screening and confirmatory purposes. Screening EIAs are performed successfully with full antigen lysates, a composition of recombinant antigens, or a mixture of both. Better performance of the assays is accomplished by of the additional use of "in vivo" antigens (Dbp(A), VlsE) which are expressed only in the infected host and get lost in repeated bacteriological culture passages in the laboratory [11]. The widely accepted format for the confirmatory approach is the immune blot (Western blot) with either recombinant or purified natural antigens separated by gel electrophoresis. Since a few years strip immune blots with sprayed antigen bands on the reaction layer have simplified the visual and automatic recognition and identification of relevant patterns.

Multiplex assays based on bead technology now open the next generation of immune assays. They are capable to cover a high number of distinct reactions specific for one single antigen each in one reaction tube. This is usually achieved by coating the antigens to distinct bead populations which can later be identified in liquid fluorescence cytometers. The combination of particle size and colour of the beads allows for the discrimination of up to 100 or even more separate reactions in a single run.

3. Bead based multiplex assays for the serodiagnosis of Lyme borreliosis

3.1. Assays and antigen composition

There are currently three suppliers of commercially available multiplex based assays for the serodiagnosis of Lyme borreliosis in Germany: PROGEN Biotechnik GmbH, Heidelberg (Multimetrix Borrelia IgG/IgM Test); MIKROGEN GmbH, Neuried (recomBead Borrelia IgG/IgM); Institut Virion/Serion GmbH, Würzburg (Multianalyt Borrelia burgdorferi IgG/IgM). All assays are intended as confirmatory tests designed for detection of IgG or IgM antibodies directed against Borrelia antigens in analogy to a Western blot. Apart from the Borrelia lysate being coupled natively to the beads, recombinant antigens are used exclusively in all three tests. All assays have to include antigens being capable to detect both early (predominantly IgM) and late immune response (mainly IgG). However, the assays differ markedly in their antigen composition. For example, Mikrogen omits the flagellin derived p41i fragment and the lysate antigens despite their strong immunogenicity, in order to avoid unspecific reactions. Virion/Serion has no OspA and might therefor miss hints at cases of Lyme borreliosis evolving with autoimmunologic complications. In all three assays, several Borrelia subspecies specific variants are provided for certain antigens (OspC in Progen and Mikrogen [important for early disease], Dbp(A)/p18 in Mikrogen and Virion/Serion tests [relevant for late disease]) to cover the broad expected spectrum of immune response in the infected host. While Mikrogen and Virion use the same antigen panel for their IgG and IgM tests, Progen prefers dedicated antigen panels for the two antibody classes in order to cover expected immune reactions concerning the p58 and the OspC antigens. A detailed list of the exact antigen composition in the different tests is given in Table 2.

There are also relevant differences in assay procedures. The Progen test is a no-wash assay designed for rather high sample dilutions. In contrast Mikrogen and Virion assay procedures prefer washing steps requiring a vacuum extraction system or especially equipped ELISA plates and washers. For the detection of the immune reaction Progen and Mikrogen exclusively utilize the Luminex™ analyzer platforms while Virion assays can also be analyzed by common flow cytometers.

3.2. Calculation of results

Regardless of the technical platform used the presence of Borrelia antibodies in the sample results in a fluorescence signal mediated by phycoerythrin conjugated detector antibodies. Mean fluorescence intensities (MFI) derived from 70 to 100 analyzed beads per antigen is used for the quantification of the immune reaction. Progen and Mikrogen assays both deliver cut-off-index (COI) based semi-quantitative results. Cut-off values necessary

Table 1
Borrelia key antigens and their relevance for serological diagnostic use.

Antigen	Description	Specificity	IgM/early	IgG/late	Remarks
VlsE	Variable major protein-like sequence, expressed	+++	++	+++	Best single antigen found so far for IgG response
OspC/p22	outer surface protein C	+++	+++	+	Strong IgM reaction in acute infection
p100	Membrane vesicle protein	+++	+	+++	Indicator of prolonged infection
Dbp(A)/p18	Decorin binding protein A	++	(+)	+++	Indicator of chronic infection
p41i	Internal part of the flagellum antigen p41	++ (IgM) + (IgG)	++	(+)	Strong immune reaction, higher specificity than whole flagellin p41
OspA	Outer surface protein A	+++	(+)	+	Indicator of severe/complicated infection (possibly autoimmune)
Lysate (full antigen)	Crude natural antigenic mixture	(+)	+	(+)	Strong immune reaction, highly cross-reactive