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The Open Dermatology Journal

Content list available at: www.benthamopen.com/TODJ/

DOI: 10.2174/1874372201610010062

Diagnostic Tools for *Borrelia* Assessment in Humans

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Abstract: Although the etiological agent of Lyme disease has been known since 1980s, diagnosis of Lyme disease is still a controversial topic because of the wide range of clinical manifestations and the limited diagnostic tools available to assess *Borrelia* in humans.

The most used diagnostic tool for Lyme disease is currently serology, but also Polymerase chain reaction (PCR) and other methods are often used to prove *Borrelia* infection in different patients' specimens. The present article deals with most of the diagnostic tools used in clinical practice for Lyme disease detection in human samples. Direct and indirect specific methods for *Borrelia* infection detection will be discussed.

The most recent peer reviewed publications as well as original results from our study and information provided by companies' web sites have been analyzed to compile this review article.

Keywords: *Borrelia*, commercial kits, PCR, serologic tests, standardization.

1. BACKGROUND

Although the etiological agent of Lyme disease has been known since 1980s, diagnosis of Lyme disease is still a controversial topic [1]. The medical community is divided into two groups: the first one recognizes that Lyme disease is associated with certain objective clinical manifestations supported by laboratory evidence of infection with *Borrelia burgdorferi* sensu lato, while the second one believes that Lyme disease patients may also present with aspecific symptoms without laboratory evidence in case of occult infections [1, 2]. Except in cases with the pathognomonic clinical manifestation of erythema migrans, the diagnosis of Lyme borreliosis usually requires confirmation by means of a microbiological diagnostic assay [3]. Although the most informative tool for *Borrelia* detection is direct culture isolation from blood or other body tissues, this approach is unsuitable for diagnosis and its use is confined to particular situations. Indirect methods for *Borrelia* infection are thus preferred, and the main diagnostic tool for Lyme disease is nowadays serology, which is the only one approved by the FDA (Food and Drug Administration). Detection of *Borrelia* by culture isolation and nucleic acid techniques is confined to special situations, such as to clarify clinically and serologically ambiguous findings [3]. Recently, CE-IVD (European Community marked –*in vitro* Diagnostic Medical Devices) tests for *Borrelia* detection by PCR in clinical specimens have been produced [4 - 10], but none has been approved by the FDA. In the following sections both serologic and PCR techniques for *Borrelia* detection will be discussed.

Over the last decade non-specific tests have also been investigated for Lyme disease diagnosis; among them, CD57 cell count [11 - 13] and chemokine 13 level in cerebrospinal fluids [14 - 16] have been proposed with controversial results. It is not the aim of this study to report on aspecific detection methods, therefore those types of analyses will not be discussed hereafter.

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2. DIRECT DETECTION OF *BORRELIA*

A variety of laboratory techniques have been developed for direct detection of *B. burgdorferi* sensu lato. Those assays provide evidence for the presence of intact spirochetes or spirochete components such as DNA or protein in tick vectors, reservoir hosts, or patients.

Four different approaches have been used in the clinical laboratory: microscope-based assays, detection of *B. burgdorferi*-specific proteins or nucleic acids, and culture [17].

2.1. Culture

Borrelia burgdorferi sensu lato can be cultivated from various tissues and body fluids in modified Kelly-Pettenkofer medium (MKP), Barbour-Stoenner-Kelly II (BSK-II) medium, and commercially available BSK-H medium [3, 18]. The yield of *Borrelia* culture from clinical samples is usually low with a higher rate of positivity from skin biopsy compared to blood and CSF cultures [18]. *Borrelia* cultivation and isolation is a time-consuming and demanding procedure. Successful cultivation can take up to 12 weeks [18]. In addition, preparation of culture media is demanding and expensive. Nonetheless, cultivation is the golden standard to prove the presence of active infection by *Borrelia.* Slow growth along with low clinical sensitivity [17, 19] do not make it a diagnostic tool for Lyme disease, and for this specific reason it will not be deeply considered in the present article.

2.2. Microscopy

Microscopic detection of *Borrelia burgdorferi* sensu lato has not been frequently used in clinical practice for confirmation of Lyme disease, mostly due to the sparseness of organisms in clinical samples [17]. Warthin-Starry stain, which consists of silver impregnation of microorganisms, was the oldest method used to search for Borrelia in histologic preparations in the 1990s, but recently only two publications have reported on the use of this stain for Borrelia detection [20, 21]. Among microscopic techniques focus floating microscopy (FFM), a modified immunohistochemical technique that combines several strategies to detect minuscule organisms in tissue sections, appears to be the most promising one for direct *Borrelia* detection, because it can be successfully applied on fresh material, nitrogen-frozen material, and paraffin-embedded tissues, in many cases on blocks older than 30 years [22]. Focus-floating microscopy has been recently proposed as the possible golden standard for detecting cutaneous borreliosis [22]. Not only a polyclonal antibody to *Borrelia*, a vivid chromogen (3-amino-9-ethylcarbazole) and the purposeful omission of counterstaining are necessary, but the technique also requires the histologist's patience [23]. By meticulously examining the entire slide horizontally and vertically, Eisendle *et al*. [22] have been able to find organisms with 98% sensitivity in classic examples of cutaneous borreliosis [22]. Since its first appearance in 2007 [22], FFM has been applied to several studies [21, 24 - 35], enabling detection of *Borrelia* and allowing that a number of dermatological conditions, such as morphea and lichen sclerosus et atrophicus, could be attributed to Lyme Borreliosis [28]. Unfortunately, although FFM is promising and helpful to prove *Borrelia* infection in cutaneous atypical lesions, only a few centers have adopted this technique.

2.3. PCR

Direct detection of *Borrelia* in clinical specimens is typically accomplished by PCR. Advantages of PCR are sensitivity, simplicity and speed [36]. A huge amount of articles have been published reporting on the use of PCR assays for *Borrelia* detection in human specimens, but large comparative studies aimed to identify the best PCR method are still lacking [3]. PCR assays differ in terms of target (borrelia sequence targeted by primers), PCR approach (nested PCR, real time PCR, PCR followed by hybridization…) as well as type of samples (blood, biopsies, cerebrospinal fluid, synovial fluid…) [37, 38]. Different types of PCR protocols could be applied for *Borrelia* detection, namely nested PCR, PCR followed by hybridization, realtime PCR, the latter both in qualitative as well as quantitative setting [37, 38]. Several and different protocols, mostly in house, are in use in most laboratories. Differences in protocols also rely on the type of samples and extraction procedures together with the broad range of clinical manifestations of Lyme disease. Different methods could be used to extract DNA and they could have different yields. Moreover, DNA extracts could be contaminated by PCR inhibitory substances due to poor extraction procedures [37]. Because of *Borrelia* paucity in biological liquids and tissues the diagnostic sensitivity of PCR was found to be low when using low volumes of sample material [3, 37]. As reported by Nolte PCR assays to detect *Borrelia* from CSF, synovial fluids or urines need large volumes for extraction in order to increase the sensitivity of the method [37]. Low and transient spirochetemia and high spirochoetes' tropism for tissues (joints, heart, meninges) may account for negative PCR results in blood and other

biological fluids [38]. While PCR is highly sensitive for detection of *B. burgdorferi* sensu lato DNA in skin biopsy samples, such testing for patients with Lyme disease involving systems other than skin, has in general low sensitivity, with the exception of patients with Lyme arthritis [17]. PCR protocols for *Borrelia* detection have been developed by targeting plasmid as well as borrelial chromosome. The most frequent target for plasmid borrelial DNA is OspA which in some cases have also been used for *Borrelia* genotyping [36, 39 - 43]. Among chromosomal targets the most frequently assessed in PCR assays are flagellin [43 - 47] and 66 kDa protein [41, 44, 48, 49] which were historically the first targets analyzed by PCR and most recently the gene encoding the *Borrelia* 16S rRNA [36, 43, 48 - 50]. As some plasmids may be present in more than a copy per *Borrelia* cell a plasmid target based PCR could be more sensitive. The "target imbalance" in *Borrelia* assays could be one of the multiple issues affecting *Borrelia* detection by PCR [37].

Overall, standardization is the major concern with respect to PCR for *Borrelia* detection.

PCR detection of *Borrelia* DNA does not enable to distinguish between dead or living microorganisms, because it is a poor indicator of the viability of the bacteria [51]. To this regard, quantitative analysis of flagellin B mRNA of *Borrelia* has been shown to correlate well with the culture results in Lyme disease, thus demonstrating the bacterial viability [51]. Although RNA analyses for *Borrelia* could be a surrogate biomarker for *Borrelia* active infection it is laborious and not suitable for routine analyses [37].

Recently, several CE-IVD PCR assays have been developed for *Borrelia* detection, among them: the EliGene®*Borrelia* kit (Elisabeth Pharmacon group, Czech Republic and UK); the *Borrelia burgdorferi* PCR kit (GeneProofa.s, Czech Republic), the real time PCR *Borrelia burgdorferi* sensu lato (Dynex, Czech Republic), the *Borrelia burgdorferi* s.l. kit (Biorn Diagnostics GmbH, Germany), the *Borrelia Burgdorferi* kit (Clonit S.r.l. , Italy), the BactoReal®*Borrelia Burgdorferi* sensu lato (Ingenetix, Austria) and the Diarella Borrelia real time PCR Kit (Gerbion, Germany) [4 - 10]. Most of them are qualitative real time PCR tests, multiplexing both an internal host control (human housekeeping gene) and the specific *Borrelia* target. Detection of DNA of *Borrelia* species pathogenic to human (*B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto) has been guaranteed by amplifying flagellin gene [5 - 7], but also OspA [9]. Those commercial kits could be used for blood, additionally tests could be applied to cerebrospinal fluid and urine [4 - 6, 9, 10], to ticks [4, 10] or ticks' suspension [6]. Sensitivities of those tests are highly variable and not conventionally described. Overall those commercial assays could have an impact on the reproducibility of PCR assays for *Borrelia* detection only because they decrease the variability in the reagent preparations and primer design. To this regard, the use of commercial kits represents a sort of harmonization in the assay preparation rather than standardization. There are no scientific publications comparing the performance of those commercial products with respect to serology, cultivation and in house PCR protocols in Lyme disease patients and controls. Clinical usefulness and added value to routine of those commercial assays should be confirmed/assessed by large validation studies with internal and external quality controls.

Overall, the scenario of PCR analyses for Lyme disease confirmation is wide and having reliable, reproducible tool standardization is of paramount importance in all the phases of the PCR process, starting from sample selection (*e.g.*, type of sample and the required volume to be processed) as well as the pre-analytical phases such as the extraction procedures.

3. INDIRECT METHODS- SEROLOGY

Antibodies against *Borrelia* species are slow to develop, with IgM generally not being detectable for the first 1-2 weeks from the infection and IgG often not emerging for 4-6 weeks [52]. Moreover, there is evidence that some patients with solitary erythema migrans (EM) as their only manifestation may never seroconvert [52], particularly in Europe [53].

In Lyme disease the rate of seropositivity seems to correlate with the duration of symptoms before diagnosis [54] and duration of treatment not only at presentation, but also during follow-up, indicating that early antibiotic treatment may abrogate the development of seropositivity [52]. There is great variability in the specific antigens recognized by individual sera and the extent to which serum antibodies recognize homologous proteins from other pathogenic *Borrelia* species or even from heterologous strains of the same species [52, 55].

Both IgM and IgG *Borrelia*-specific antibodies may persist for years in some patients [1], which makes it impossible to distinguish between past and newly acquired infections based on seropositivity alone [52]. Seroprevalence indeed is of particular interest in endemic areas, showing in some European regions a gender and age trend [13, 56, 57].

In Europe [58], United States [59] and Canada a two tier testing serology is recommended for borreliosis

assessment[1, 2, 60]. The first tier consists of a sensitive initial serological test or tests that detect class-specific antibodies (IgM and IgG, either together or separately). First-tier tests are enzyme immunoassays (EIAs) such as ELISAs or, rarely today, indirect immunofluorescence assays (IFAs) as they require a skilled microscopist and cannot be scored objectively. If the result of first-tier testing is negative, the serum is not tested further. If the result is positive or indeterminate (a value that is sometimes called 'borderline'), a second step should be performed [60]. The second tier determines the antigens recognized by the antibodies detected in the first step using separate IgG and IgM immunoblots.

General limitations in serological tests for Lyme borreliosis are related to detectability of antibodies, to differences in *Borrelia* genospecies and to the clinical spectra of the Lyme disease. It is well known indeed that two-tiered testing is insensitive in acute erythema migrans and may be negative in early neuroborreliosis [1, 2, 52, 60].

General drawbacks for two tier approaches are that the two-step procedure is complex, technically demanding and costly. Besides the technical aspects of the serological tests, Perronne [2] has reported on several other factors that could lead to seronegativity in confirmed cases of Lyme disease: among such factors are sequestration of antibodies in immune complexes, different species and subspecies of *Borrelia* distributed worldwide, as well as coinfections with other pathogens transmitted by the same vector.

Taken those observations it is manifest that new or better standardized immunological methods are needed to support the diagnosis of Lyme disease.

3.1. ELISA

Most commonly in ELISA, antigen mixtures composed of whole-cell sonicates of *B. burgdorferi* sensu lato are used as the source of antigen for the the individual or in combination detection of IgG, IgM, or IgA antibodies. Usually those sonicates are derived from strain B31 [2], a specific strain of *Borrelia burgdorferi* sensu stricto, which is the only organism established to cause Lyme disease in North America [60]. Since in Europe and Asia Lyme disease also results from infection by *Borrelia garinii* or *Borrelia afzelii*, as well as by the recently described *Borrelia spielmanii* in Europe; diagnostic tests using antigens from *B. burgdorferi* sensu stricto will not necessarily perform well for infections carried by other genospecies, although some do [60].

The Vmp-like sequence expressed (VlsE) protein is a surface-exposed lipoprotein encoded by a linear plasmid of *B. burgdorferi* B31 and it has been found to be highly immunogenic [17]. In the last decade, an ELISA using only a single synthetic peptide derived from the VlsE sequence (IR6 or C6 peptide) as the source of antigen has become commercially available and is quite often used in the first step of the serological analyses. Both the single C6 peptide and whole VlsE assays have been approved as alternatives to whole-cell ELISAs as first-tier tests [60]. In addition, the Immunetics C6 test (Immunetics Inc, MA, USA) has recently been evaluated as an assay that could be used in place of both steps of two-tiered testing, that is, as a simple 'stand-alone' test [60]. C6- and VlsE-based assays have the additional feature of detecting antibodies to Eurasian genospecies of *Borrelia* (*i.e*. *B. garinii*and *B. azfelii*) as well as *B. burgdorferi* sensu stricto [60]. Moreover, in a multiplex bead based assay for the detection of serum antibodies to *Borrelia burgdorferi* sensu lato it has recently been shown that VlsE IgG had the highest diagnostic value even in patients with neuroborreliosis [61].

3.2. Immunoblots

Regarding immunoblots, recommendations have been published by Centers for Disease Control and Prevention (CDC) on the number and types of IgM and IgG bands in order to consider immunoblot results as positive [59]. However, those rules could be applied in the US, but not to patients infected in Europe since immune response of European patients is restricted to a narrower spectrum of *Borrelia* proteins, compared with that shown by American patients [3].

Consequently, the existence of at least 3 pathogenic species requires species-specific interpretation rules [52].

Traditional blots are hard to standardize, as they are only semi-quantitative and reading them involves often subjective judgment about the significance of weak bands [60].

4. OUR EXPERIENCE

As an explicative example of the assumption that both PCR analyses on DNA and serology do not allow distinguishing between active and inactive *Borrelia*, here we report our (unpublished) data referred to serological and PCR analysis of healthy volunteers living in an endemic area. All the participants have lived in villages of the

transborder rural area between Italy and Slovenia and all of them reported having been bitten by ticks during their life. All the participants in the study completed a questionnaire designed by the author of the presented study, and had both serologic tests performed using immunoenzymatic test VlsE ELISA, by which the level of *Borrelia burgdorferi* IgM and IgG class antibodies were determined in blood serum as well as two PCR analyses on blood extracts targeting a fragment of flagellin gene and 66 kDa protein [44]. Blood and data were collected over May 2005. Fifty-two people participated in this survey (median age 47 years), 31 of them were males and 21 females. Of those, 8 participants (5 males and 3 females) had had Lyme disease in the past. Among the requested information, participants reported the date of their last tick's bite, the therapy followed for concurrent chronic diseases, their job and if they had taken antibiotics in the month before blood and data collection. Results and participants' characteristics are reported in (Table **1**). Overall there are no differences in the antibody profiles between volunteers with previous Lyme disease or without. Both IgG and IgM positivity and detection of *Borrelia* by PCR were not related to previous antibiotic treatment (p=1.0 for both) as well as to concomitant chronic disease (p=1.0). IgG and IgM resulted simultaneously positive only in one participant, a 67-year-old man who didn't have Lyme disease previously. The only volunteer who had positive *borrelia* detection by PCR (flagellin) was a 26-year-old man who reported Lyme disease (erythema migrans) 1 month before. In that person IgG and IgM profiles were negative. All volunteers with positive IgG or IgM underwent dermatological examination (PIC Interreg IIIA Italy-Slovenia 2000-2006 Project No: AAFVG332366). None of the seropositive volunteers had signs of Lyme disease.

Table 1. Data on participants.

	With previous Lyme disease (8)	Without previous Lyme disease (44)	*p*
Gender Female Male	5 3	18 26	0.9
IgG positive	4	11	0.2
IgM positive	1	7	0.6
PCR flagellin positive	1	0	0.2
PCR 66 kDa positive	0	0	1.0
Job Clerical or educational workers Retirees Students	3 3 2	26 11 6	0.5

Overall both IgG and IgM positivity, as well as PCR, were not associated with any of the requested information although IgG positivity tends to prefer older volunteers (p=0.08). IgG positivity did not result significantly different among jobs, but IgM (p=0.04) was detected in 0 out 29 workers, in 1 out of 8 students and 3 out of 14 pensioners.

Our data agree with the scientific community [1, 60] regarding the usefulness of both PCR and serologic exams for Borrelia detection in healthy people.

CONCLUSION

As a general consensus diagnostic investigation for Lyme disease should be restricted only to those people who show signs and/or symptoms of Lyme disease. In endemic area serologic positivity could be a quite a frequent event because of the exposition to *Borrelia*. Diagnostic tests are of clinical value only if they are used properly in patients with objective signs of Lyme disease and with a history of potential exposure to infected vector ticks [60].

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The author is grateful to Dr Valentina Melita for the language revision of the manuscript and to Dr Ermanno Nardon for his critical suggestions.

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Received: October 22, 2015 Revised: December 16, 2015 Accepted: December 16, 2015