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REVIEW

Progress in the molecular diagnosis of Lyme disease

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ABSTRACT

Introduction: Current laboratory testing of Lyme borreliosis mostly relies on serological methods with known limitations. Diagnostic modalities enabling direct detection of pathogen at the onset of the clinical signs could overcome some of the limitations. Molecular methods detecting borrelial DNA seem to be the ideal solution, although there are some aspects that need to be considered.

Areas covered: This review represent summary and discussion of the published data obtained from literature searches from PubMed and The National Library of Medicine (USA) together with our own experience on molecular diagnosis of Lyme disease.

Expert commentary: Molecular methods are promising and currently serve as supporting diagnostic testing in Lyme borreliosis. Since the field of molecular diagnostics is under rapid development, molecular testing could become an important diagnostic modality.

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1. Introduction

Lyme borreliosis (disease) is caused by spirochete of the *Borrelia burgdorferi* sensu lato complex. Among at least 21 delineated species, 5 have been isolated from humans and described as human pathogens in Europe, *Borrelia afzelii, Borrelia garinii, Borrelia bavariensis, Borrelia burgdorferi* sensu stricto, and *Borrelia spielmanii*, whereas *B. burgdorferi* sensu stricto presents the main causative agent of the human disease in the North America [1,2]. Recently, *B. bissettii* and *B. mayonii* were described as the cause of Lyme borreliosis in USA and Canada [3–5].

Based on average nucleotide identity and phylogenetic analysis Adeolu et al. proposed separation of the genus *Borrelia* into novel genus *Borreliella* gen. nov, containing the causative agents of Lyme borreliosis and emended genus *Borrelia*, containing the causative agents of relapsing fever [6]. To avoid confusion in already complicated taxonomy of borreliae, we used previous familiar terminology (genus *Borrelia*) while the terms borrelia and borreliae refer to Lyme disease group of spirochetes.

B. burgdorferi sensu lato survives in an enzootic life cycle consisting of arthropod vectors and various vertebrate hosts and has great potential to adapt to various microenvironments found in mentioned biological niche [7,8].

Ixodes ticks are the main vectors of *B. burgdorferi* sensu lato, mainly *Ixodes ricinus* in Europe, *Ixodes persulcatus* in Asia, *Ixodes scapularis* in northeastern and upper midwestern USA, and *Ixodes pacificus* in western USA [8,9]. Ixodes ticks acquire spirochetes during their blood meal. Once infected, ticks retain borrelia for a long time, even between molts, effectively transmitting spirochetes to the next feeding stage and/or to a host. Because a single tick consumes vertebrate blood multiple times, infection with more than one *Borrelia* species has been observed in ticks [7]. In humans, a feeding period of more than 36 h is usually required for borrelia injection with tick saliva but this is not a fixed rule [8,10–12].

Many environmental factors like size of a tick population, vertebrate density, climate changes, vegetation cover as well as dominance, maintenance, or disappearance of specific *Borrelia* species can influence the interaction between ticks and pathogens [7]. This complex biological interaction between pathogen and host is based on discrete molecular processes that represent the main focus of ongoing research studies.

Reservoir host of borrelia are wild animals, birds, and lizards. Different host species vary in their ability to acquire borreliae from infected ticks, to harbor borrelia, and act as long-term source of spirochaetes [7,9]. Wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), and bank vole (*Clethrionomys glareous*) were identified as principal reservoir hosts of *B. burgdorferi* sensu lato in Europe, but dormice, hedgehogs, rats, squirrels, hares, and others were also described [13,14]. Distinct *Borrelia* species are associated with different reservoir hosts: *B. afzelii* and *B. bavariensis* with rodents, *B. garinii* and *B. valaisiana* with birds, *B. spielmanii* with dormice, while *B. burgdorferi* sensu stricto does not seem to be associated with specific reservoir host [7,15,16]. Borrelial infection of the host is lifelong despite the presence of specific immunity and is generally not harmful to the host [15]. Many studies are currently based on molecular and genetic analysis trying to elucidate borrelial sensing of the surrounding environment, their alternation of gene expression and rapid adaption to a new host [7,15].

The *B. burgdorferi* sensu lato genome consists of a linear chromosome (of approximately 910 kb) and numerous linear and circular plasmids (comprising up to 40% of the genomic

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DNA) [17]. Analysis of extrachromosomal DNA shows that there is considerable heterogeneity among strains in their plasmid profiles; strains differ in the number and size of plasmids. Although the majority of housekeeping genes are located on the chromosome, genes encoding different lipoproteins as well as features largely involved in virulence and interaction with hosts are located on the plasmids [17]. The organization of ribosomal genes in *B. burgdorferi* sensu lato genome is unique – strains possess a single gene encoding 16S rRNA (*rrs*) and tandem repeated gene pair of 23S (*rrlA* and *rrlB*) and 5S rRNA (*rrfA* and *rrfB*) separated by a non-coding region [18]. This unique rRNA gene and a variety of other target genes [flagellin, VlsE, outer surface proteins (OspA, OspC, OspB), *hbb* gene, etc.] were used for *Borrelia* sp. analysis [18].

Once transmitted from tick to host, borreliae colonize their target tissue and the infection most frequently results in skin manifestation, erythema migrans (EM), from which borreliae disseminate. Disseminated borreliae may cause multiple erythema migrans, Lyme neuroborreliosis, Lyme carditis, or borrelial lymphocytoma while persistent (chronic) infection can be manifested as acrodermatitis chronica atrophicans (ACA), chronic Lyme arthritis or late neurological complications [2,19]. In Europe, association of particular *Borrelia* species with clinical presentation of infection indicates that *B. afzelii* is mostly associated with skin manifestations with few systemic symptoms, *B. garinii* with nervous system infections while *B. burgdorferi* sensu stricto seems to be the most arthritogenic and could cause more systemic symptoms although all species can cause erythema migrans [2]. In the North America, different ribosomal RNA intergenic spacer types (RST) of *B. burgdorferi* sensu stricto were found to vary in inflammatory potential and clinical manifestations of Lyme borreliosis [20]. The background of the Borrelia–host interaction is very complex and has been the subject of intensive study for the last 25 years. Molecular methods have helped gain substantial knowledge on the adaptation mechanisms to tick vectors and mammalian hosts, host response and immune evasion [21].

Lyme borreliosis is the most common tick-borne disease in Northern Hemisphere. Yearly incidence rates in Europe range from 0.001 cases per 100,000 inhabitants in Italy (2001–2005) to 188.7 cases per 100,000 in Slovenia in year 2014 [22,23].

According to Centers for Disease Control and Prevention (CDC), the number of confirmed cases in the USA was 25,359 in 2014 (7.9 cases per 100,000), 96% of confirmed Lyme disease cases were reported from 14 states, concentrated heavily in the northeast and upper Midwest. Recently, Nelson et al. estimated that annual incidence of Lyme borreliosis is 106.6 cases/100,000 persons and that over 300,000 cases occur annually [24].

Although the Garin–Bujadoux–Bannwarth syndrome is defined as a typical manifestation of Lyme borreliosis, a clinical diagnosis of borrelial infection can be made by the clinician based only on the pathognomonic borrelial rash erythema migrans. For all other clinical presentations, where signs and symptoms are more or less associated with borrelial infection, the diagnosis should rely on laboratory confirmation [19,25,26].

The majority of laboratories perform tests based on the detection of specific borrelial antibodies in the serum. Serological confirmation can be challenging due to antigenic complexity of different *Borrelia* species, differences in immune potential of borrelial antigens and the specific patient's immune response. Several approaches have been used to improve specificity and sensitivity including two-tier algorithm testing, use of recombinant antigens and different serological methods [9,19,25,27]. Sensitivity of serological assay differs depending on the clinical picture; in acute sera of patients with erythema migrans sensitivity is relatively low, around 50%, whereas in the case of disseminated infection it is higher [25,28].

The microbiological diagnosis of most bacterial infections is based on *in vitro* culture and identification of the causative microorganism but despite attempts to improve sensitivity, and simplify the procedure, borrelial culture is not a routinely available diagnostic method. The sensitivity of culture correlates with the number of organisms present in samples which appears to be quite low in borrelial infection [25,27]. The rate of positive cultures of skin biopsies, blood, and cerebrospinal fluid (CSF) are 40–60%, 5%, and 10%, respectively [25,29–32]. Higher rates, 40%, were reported from high-volume cultures of 9 ml plasma [33,34]. Because of fastidious and long-lasting procedure, many laboratories try to overcome the inherent disadvantages of cultures with molecular methods [25,27]. For isolated strains, molecular-based typing enables discrimination between distinct isolates and could assist in the clarification of relationship between pathogen and its hosts and vectors, moreover, can contribute to define borrelia ability to cause different clinical manifestations in humans [35].

2. Molecular methods in Lyme borreliosis

Molecular methods include all the techniques for detecting and/or analyzing nucleic acid. Among all, polymerase chain reaction represents the most often used technique.

Molecular methods can (1) assist in the confirmation of Lyme borreliosis, (2) serve as methods for identification and typing borrelia directly in specimens or cultured isolates, (3) enable detection of borrelia in reservoir hosts or tick vectors, and (4) enable detection of coinfection with different *Borrelia* species in particular sample (ticks, reservoir hosts, humans).

2.1. Molecular methods for confirmation of Borrelia infection

In order to detect *Borrelia burgdorferi* sensu lato in clinical material, mainly PCR-based methods are used. Diagnostic sensitivity and specificity of PCR are very important parameters that are influenced mainly by clinical picture, appropriate clinical specimens, sample collection and DNA extraction, determined target gene for amplification, applied PCR method (standard one-step, nested or real-time PCR), and presence of contaminants and/or inhibitors.

For determining the sensitivity of PCR, culture of pathogen is required. However, in Lyme borreliosis, culture often fails as a gold standard. Moreover, the clinical picture of borrelial

infection (with the exception of erythema migrans) is inconclusive and is also a poor choice for a comparative standard.

Appropriate clinical specimens regarding clinical presentation and stage of the disease should be collected: skin biopsy from the periphery of erythema migrans and ACA, blood in disseminated infection, CSF when suspecting infection of the central nervous system (Lyme neuroborreliosis) and synovial fluid in Lyme arthritis. Urine is not an appropriate specimen, since reported sensitivity and specificity are highly variable and several guidelines advise against its use [9,25,36].

Due to the low borrelial load in clinical material special attention to sample collection, amount of the sample, transport, and storage should be applied. While a sufficient amount (>1 ml) of clinical sample is preferred, obtaining a high volume of some specimens can unfortunately be difficult to obtain. To overcome the problem of sample quantity, the protocol for DNA extraction must be optimized to ensure a sufficient yield of borrelial DNA: clinical samples, ticks liquid samples can be concentrated by high-rpm centrifugation, solid tissues homogenized, and treated with proteinase K; all these procedures can increase the sensitivity of PCR but also the contamination risk.

Clinical samples typically have overwhelming ratio of human to pathogen DNA with low pathogen concentration. Large amount of human DNA may outnumber bacterial DNA and also have inhibitory effect. There are some commercial kits that try to overcome this problem by enabling enrichment of microbial DNA in the sample. One of these, MolYsis Basic5 kit (Molzym, Bremen, Germany), selectively lyses human cells using chaotropic reagents, and degrades any released DNA with DNase prior to the extraction of pathogen DNA [37]. Pathogen DNA originating from live cells is preserved, but extracellular DNA from dead pathogens is removed. A limitation of the method is the possibility of pathogen DNA being degraded in bacteria with a thin cell wall, those without a cell wall or those exposed to cell wall-active antibiotics and/or human immune system [38].

Another method, NEBNext Microbiome DNA Enrichment kit (New England Biolab's, USA) separates vertebrate DNA from microbial DNA on the basis of differences in CpG methylation abundance; eukaryotic DNA has higher CpG methylation rates. By using the methylated CpG-specific binding protein MBD2, human DNA is selectively bound and separated. Protein MBD2 is fused to a Fc fragment of human IgG, the latter one to Protein A-bound magnetic beads that enable simple extraction [37,39].

In the study of Thoendel et al., both enrichment kits were compared on spiked uninfected sonicated fluid resulting in 6-fold enrichment of bacterial DNA with the NEBNext kit and 76-fold enrichment with the MolYsis kit [37].

The third approach, Pureprove (SIRS-Lab GmbH, Jena, Germany), uses a DNA binding protein that recognizes unmethylated CpG motifs predominantly present in bacterial genomes. Since CpG islands and motifs are not distributed equally over the entire human genome and fragments of human genome without 5'-methylcytosin are present, efficiency of the method can be affected [38,40]. Horz et al. compared the efficiency of MolYsis and Pureprove; both protocols substantially reduced the human background DNA; however, complete elimination of human DNA was more often achieved with MolYsis, but also loss of bacterial DNA was larger with MolYsis [38].

Extraction can either be performed manually or automatically. Automated methods are generally just as effective as manual methods [41], the main advantage of automatization being a short hands-on time. When deciding for automated system, one must consider the number of samples to be processed, sample types, setup time, and run time [42].

There are many available automated methods suitable for processing up to 32 samples of several types in approximately 1 h, for example NucliSens easyMAG (bioMerieux), MagNA Pure Systems (Roche), QIAcube (Qiagen), EZ1 (Qiagen), Maxwell (Promega) [42].

The crucial element of molecular methods is the selection of an appropriate target DNA sequence to be amplified. The target must be genetically stable and should enable the detection of all species in the *B. burgdorferi* sensu lato complex [43]. PCRs targeting numerous *Borrelia* genes have been employed for research purposes but only few have been widely used for PCR detection in clinical samples, for example p66, 16S rDNA gene, 23S rDNA, 23S–5S rDNA intergenic space, *ospA, ospB, ospC, fla, dbpA, recA, bmpA* [25,44]. Target genes are located either on the chromosome (*hbb, fla* gene, 16S rRNA gene, 23S rRNA, 23S–5S rRNA intergenic space, *recA, bmpA*) or plasmid (*ospA, ospB, ospC, dbpA* gene) in different number of copies, mostly as single copy, some as two copies, for example 23S RNA, and 23S–5S intergenic space [45].

Some of the genes, 16S rRNA gene, *ospA, fla*, and *recA* are targeted by several commercially available PCR kits, mostly qualitative real-time PCR assays.

Some borrelial target genes have low discriminatory power to distinguish *Borrelia* species (like *fla* gene used in some commercially designed assays), while others, located on plasmids, are highly variable (*ospA, ospB*, and *ospC*) and therefore amplification may not occur in all strains [46,47]. In Europe where several *Borrelia* species are present, identifying *Borrelia* species in particular human sample would be preferable with regard to clinical manifestation. Currently, several post-PCR analyses are possible for *Borrelia* species identification including restriction, sequencing, hybridization, single-strand conformation polymorphism, and melting temperature analysis [48–51].

To improve sensitivity, studies with simultaneous usage of up to eight targets were published [52,53]. In the study of Eshoo, isothermal amplification of the eight borrelial target regions was combined with electrospray ionization mass spectrometry. Authors reported a 62% sensitivity in blood of patients with early Lyme disease [53]. Multiplex real-time PCR targeting 5S–23S rRNA intergenic space, *ospA* gene and *flaB* gene was found to be four times more sensitive than single target OspA PCR [52]. Further studies are required to define clinical usefulness of approaches with simultaneous usage of multiple targets and to assess whether increased costs are justified.

Different PCR assay formats can be used yielding qualitative (conventional PCR and nested PCR) or quantitative (competitive PCR and real-time PCR) results. Classical nested PCR is superior in both sensitivity and specificity to a standard PCR,

but the technique is much more prone to external DNA contamination. To avoid PCR contamination and amplicon carryover, samples should be processed in separate rooms. Real-time PCR allows the detection of PCR amplification during the early phases of the reaction and also makes quantitation of DNA more precise [54]. For detection of PCR products in real-time PCR, two common approaches are employed, the use of nonspecific fluorescent dyes that intercalate with any double-stranded DNA and the use of sequence-specific DNA probes labeled with a fluorescent reporter. SYBR Green and BEBO are nonspecific intercalation probes with the advantage of sensitive binding to double-stranded DNA, which makes them relatively easy to use and inexpensive since primer designing and optimization are not required. However, lack of specificity of these probes limits their use [55]. Hibridization probes are based on the principle of fluorescence resonance energy transfer and are sequence specific. Three common types of hybridization probes include hydrolysis probes, dual-hybridization probes, and molecular beacons [55]. Inclusion of standard curve enables estimation of the amount of the target present in initial sample. Quantitative detection of *B. burgdorferi* sensu lato in clinical samples, ticks, or reservoir hosts was determined in studies by O'Rourke et al., Stupica et al., Liveris et al., and Wilhelmsson et al. [30,56–58]. Irrespective to *Borrelia* species, studies performed on clinical material reported that larger number of borrelia cells was significantly associated with culture positive biopsies and severity of symptoms [30,56,57]; in study on ticks, the number of borrelia cells was significantly higher in adult ticks than in nymphs [58].

Inhibition of PCR may appear with the same frequency as contamination. Inhibitors can be present in various clinical samples as plasma, CSF, skin biopsies, etc. In addition, some components of common laboratory collection devices (heparin, formalin) are known inhibitors of PCR [59]. Inhibition (or internal) controls added directly to the specimen are often used in order to detect inhibition associated with the specimen matrix or the processing method [59]. Dilution of the extracted DNA could minimize problems with inhibition [60].

Since PCR does not allow distinction between living and dead organisms, positive PCR result does not prove an active disease, but the method is useful when dealing with clinical specimens from patients who recently received adequate antibiotic therapy, since PCR enables detection of the DNA of destroyed pathogen.

Lack of standardization in the sample preparation, target genes, detection methods is one of the major concerns, and also one of the reasons against the use of PCR in routine diagnostics of Lyme borreliosis. PCR methods need to be precisely evaluated before implementing in human diagnostics. Once implemented can attribute to diagnosis of borrelial infection: in early infection can confirm borrelial etiology before antibody response occurs, in ongoing infection can serve as supporting diagnostic testing and also enable determination of pathogen burden and/or pathogen identification.

2.2. Applicability of PCR in different clinical manifestations

The main advantages of molecular methods are direct detection of the agent before specific antibodies appear, identification of *Borrelia* species responsible for the infection as well as delineation of more than one *Borrelia* species in mixed infections that may be expected in some samples (e.g. skin).

2.2.1. Erythema migrans

The median sensitivity of PCR for the detection of specific borrelial DNA is high. Table 1 summarizes sensitivity, specificity, and number of included patients in 28 studies published in MEDILINE-indexed periodicals during the years 1991–2015, majority of studies were published in years 1991–2000. The median sensitivity appears higher in European studies in comparison to USA studies. One of the recent studies evaluated relationship of borrelia burden in skin of patients with EM and the disease course and post-treatment outcome. One hundred and twenty-one adult patients were included, borrelial DNA was detected in 77.7% and borreliae were isolated in 55.1% [56]. They concluded that higher borrelia burden in skin biopsy specimens was associated with a higher chance for constitutional symptoms accompanying EM and that patients with higher borrelia burden were more likely to have incomplete response [56].

Table 1. PCR from clinical specimens: sensitivity, specificity, and number of included patients in studies published in MEDILINE-indexed periodicals during the years 1991–2016.

Clinical specimen	No. of studies	No. of patients	Targets	Median sensitivity (range)	Specificity	References
Skin biopsy – EM	28	5–758	p66, 23S rRNA, flagellin, rrf-rrl, ospA, recA,	68 (30–89)	98–100	
Europe	19	5–758	16S rRNA, OspC	70 (30–80)		56, 77-93
USA	9	23–139		59 (33–81)		57, 94-101
Skin biopsy – ACA	13	5–59	p66, ospA, chromosomal DNA, 23S rRNA, rrf-rrl, flagellin	75 (20–100)	100	77, 81, 82, 84, 88-91, 102-106
CSF	22	8–190	chromosomal DNA, ospA, flagellin, rrf-rrl, 16	22.5 (5–100)	99–100	
Europe	16	8–190	SrRNK, p66	18 (9–100)		32, 52, 80, 102, 107-118
USA	6	12–81		40.5 (5–93)		119-124
Synovial fluid	12	4–124	rrs-rrl, ospC, ospA, p66, flagellin	77.5 (23–100)	100	
Europe	7	4–20		72 (23–100)		114, 125-130
USA	5	7–124		85 (60–100)		120, 124, 131-133
Blood, serum or plasma	11	7–557	polC, OspA, 16S rRNA, rrf-rrl, rpoC	18 (0–100	95–100	
Europe	5	10–557		16 (3.1– 00)		32, 134-137
USA	6	7–76		29 (0–62)		138-141

EM: Erythema migrans; ACA: acrodermatitis chronica atrophicans; CSF: cerebrospinal fluid.

Although diagnostic sensitivity of PCR in skin biopsies from erythema migrans is usually high, PCR together with culture and serology is primarily used in research studies, since the diagnosis is made on the basis of history and visual inspection of the skin lesion [9,28].

2.2.2. Acrodermatitis chronica atrophicans

The number of studies regarding PCR in ACA is lower in comparison to EM PCR studies and due to etilological agent, *B. afzelii*, studies are restricted to Europe. The median sensitivity, originating from 14 studies, is high (Table 1). Nevertheless, beside clinical presentation, diagnostic approach in the case of ACA includes almost always positive histologic findings and serology; molecular methods serve for supporting diagnostic testing, but are mostly used in research studies [9,28].

2.2.3. Lyme neuroborreliosis

Laboratory diagnosis of Lyme neuroborreliosis is based on CSF analysis that is unfavorable for the patient. Pleocytosis and intrathecal synthesis of specific borrelial antibodies occur with the course of infection. Molecular methods can be used as supporting diagnostic testing, but their main limitation is low diagnostic sensitivity [9,28]. Median sensitivity of PCR is 22.5%, there are differences between continents; median sensitivity in European studies is lower than is USA studies (Table 1).

2.2.4. Lyme arthritis

Generally, PCR analysis of synovial fluid in patients with Lyme arthritis is more sensitive in comparison to culture, median sensitivity originated from six US studies is 85%, sensitivity reported in Europe is lower, 72%, and European's studies also included fewer number of patients (Table 1).

In patients with Lyme arthritis, high concentrations of specific IgG antibodies can be detected in the serum, PCR can be used as supporting diagnostic testing [9,28].

3. Molecular methods in *Borrelia* identification and typing

Genotyping of *B. burgdorferi* sensu lato strains can assist in resolving issues in epidemiological, clinical, and evolutionary studies. Numerous methods differing in approach and targets are available for *Borrelia* species genotyping, all of them are based either on whole genome typing (species identification, plasmid profile analysis, whole genome sequencing [WGS]) or PCR-based typing (sequencing, restriction, Tm determination of PCR product, etc.). Some typing methods are currently widely used while others (e.g. WGS) are still in development [1,2].

3.1. Restriction analysis of whole genome and plasmid profiling

Large restriction fragment pattern (LRFP) is based on whole genome restriction analysis using different restriction enzymes (*Mlu*I, *Apa*I, *Kpn*I, *Sma*I, *Xho*I) [142]. Species identification can be accomplished with separation of restricted genomic DNA fragments using pulse-field gel electrophoresis (PFGE). *Mlu*I–LRFP method was found to be suitable for *Borrelia* species identification and delineation of subgroups within the species. Since method is labor-intensive and requires growing borrelia culture, there are limited studies on the subject [49,142–144]. *B. afzelii* isolates show quite homogeneous restriction pattern; the majority of isolates belonging to *B. afzelii* Mla1 subgroup (>99%), and a minority to *B. afzelii* Mla2, Mla3, and Mla4 [49]. *B. garinii* and *B. burgdorferi* sensu stricto isolated strains have very heterogeneous restriction patterns and are divided into 7 (Mlg1–7) and 15 (Mlb1–15) subgroups, respectively [49]. *Mlu*I–LRFP analysis also enables delineation of strains within species *B. spielmanii, B. valaisiana*, and *B. lusitaniae* but cannot distinguish *B. bavariensis* from *B. garinii* [49].

As for *Mlu*I–LRFP, growing borrelial culture is also required for plasmid profile determination. Only linear plasmids can be visualized by PFGE, which diminishes the applicability of the method [49,145–148]. Because some plasmids can be present in low-copy number, which is below the sensitivity of PFGE, they are not detected by the method. Strains also harbor more plasmids of the same size (more replicons) which cannot be distinguished by plasmid profiling [149]. Generally, borrelial plasmids are stable and present nature make-up of the strain so plasmid profiling can be most useful method for distinguishing strains within particular species like strains of *B. afzelii*, the most frequently isolated species in Europe, which is homogeneous in *Mlu*I–LRFP analysis, and very heterogeneous in plasmid content [49].

In general, methods based on PFGE have high discriminatory power for linear DNA molecules, providing an excellent approach for species, subspecies, and clone identification [150].

3.2. WGS-based typing

Over the past decade, next generation sequencing (NGS) has become a part of routine and research methods. The most widely used application in NGS is WGS, which enables the most comprehensive view of genomic information and associated biological implications [151,152]. A comprehensive review evaluating various NGS approaches and recent advances was written by Goodwin et al. in 2016 [152]. With the evolution of NGS technologies several advances have been incorporated as longer read lengths, reduced costs, and rapid sequencing which also enabled the use in clinical diagnostics. Regarding detailed genomic information, WGS can be applied to pathogen identification, typing for epidemiological surveillance and outbreaks, resistance detection, and virulence genes profiling [153].

As all other pathogens also borrelia have undergone WGS analysis; data are available (NCBI Genome; http://www.ncbi.nlm.nih.gov/genome). Not many *Borrelia* species are reported in the gene bank library; as of August 2016 10 *B. afzelii*, 40 *B. garinii*, and 42 *B. burgdorferi* sensu stricto strains genomes are available. Comparative studies on whole DNA level, and/or typing and virulence profiling are based on culture isolates or performed directly in biological samples, nevertheless they are rare and still developing [154]. In field samples borrelial small genome is overwhelmed by the genome sizes of their vectors or hosts. Shotgun approaches for WGS of pathogen

directly form field samples were inefficient [155]. In order to enrich *B. burgdorferi* sensu lato DNA in complex samples different approaches were used. Leichty and Brisson proposed the use of selective whole genome application, which was validated on artificial mixtures of bacterial DNA [156]. More recently, Carpi et al. successfully sequenced 30 *Borrelia burgdorferi* sensu lato genomes directly from arthropod vector using multiplex hybrid capture enrichment prior WGS [157]. Authors concluded that used methodology demonstrated as highly scalable and cost-effective and could also be applied to broader applications in molecular ecology [157].

3.3. PCR-based typing

Many PCR-based molecular techniques targeting single genes are widely used for *Borrelia* species typing and are more or less successfully applied in clinical diagnosis of borrelial infection while PCR-based technique termed multilocus sequence typing (MLST) which targets several genes was developed and has great potential for defining relationships of bacterial populations [48,51,158,159].

3.3.1. PCR-based RFLP analysis of rrs-rrlA (16S–23S rRNA) spacer locus

Amplification of *rrs-rrlA (*16S–23S) spacer locus results in a PCR product of about 940 kb followed by restriction either with *Hinf*I or *Mse*I restriction enzyme and fragments separation by gel electrophoresis [160,161]. The method was applied to North American *B. burgdorferi* sensu stricto strains showing that particular RST correlate with pathogenic potential of borrelia strains; patients infected with RST1 strains had more severe symptoms, multiple erythema lesions, and more often hematogenous dissemination of the pathogen compared to patients infected with other RST [160,161]. Recently, European *B. burgdorferi* sensu stricto strains from Slovenia were analyzed and compared to American strains [162]. RST1 type was found to be dominant among Slovenian isolates (72%). These findings did not support correlation between *B. burgdorferi* sensu stricto RST1 and severe clinical manifestations because clinical presentations in Slovenian patients differed from American. Nevertheless, the study contributed significantly to our knowledge of *B. burgdorferi* sensu stricto strains from both continents – Slovenian *B. burgdorferi* sensu stricto strains vary in virulence, inflammatory potential, and clinical manifestations of infection compared to North American strains [162].

3.3.2. PCR-based RFLP analysis of rrfA-rrlB (5S–23S rRNA) intergenic spacer

PCR-based restriction fragment length polymorphism (RFLP) of *rrfA-rrlB* (5S–23S rRNA) intergenic region is the most frequently used method for *Borrelia* species typing either for cultivated spirochetes (one step PCR) or uncultivated spirochetes from clinical, reservoir host or tick samples (nested PCR due to the low number of spirochetes). The amplicon of about 250 bp is restricted by two enzymes, *Mse*I and *Dra*I resulting in RFLP of eight distinct species of *B. burfdorferi* sensu lato [51]. The method is applied in many research and diagnostic laboratories due to its sufficient discriminatory potential and high reproducibility.

3.3.3. PCR-based outer surface protein C (ospC) analysis

Gene *ospC*, located on single-copy circular plasmid cp26, is the most genetically diverse locus of *B. burgdorferi* sensu lato; in *B. burgdorferi* sensu stricto strains, for example, 28 *ospC* alleles have been identified. Great dispersion of *ospC* alleles regarding biological source of borrelia strains (ticks, humans, mammals) and geographic location (Europe, North America) was demonstrated by *ospC* genotyping [159,163,164]. The study of Cerar et al. comparing North American and Slovenian *B. burgdorferi* sensu stricto strains supports this diversity [162]. *OspC* typing is DNA sequencing-based method that requires time, equipment, and trained personnel.

OspC protein itself displays the highest amino acid variability in the surface expressed domain. While the same OspC serotypes (defined by monoclonal antibodies) can be dispersed in many geographic areas in spite of different host species composition, particular OspC serotype may infect divergent vertebrate species [165,166]. Because of complex and fastidious OspC serotyping and/or genotyping, analysis based on OspC protein are more or less restricted to reference laboratories.

3.3.4. PCR-based flagellin typing

Flagellin gene is one of the most frequently used targets for confirmation of borrelial infection in different samples (humans, ticks, and reservoir hosts); designed properly can also be used for *Borrelia* species identification [167,168]. Although Jaulhac et al. described differentiation of seven *Borrelia* species (*B. garinii, B. afzelii, B. burgdorferi* sensu stricto, *B. japonica, B. andersoni, B. valaisiana, B. bissettii)* based on flagellin gene, identification method is technically demanding and not widely used [169]. Many commercial PCR kits include this gene for diagnostic purpose because of high sensitivity of the method to detect borrelial DNA in sample [167,168].

3.3.5. Real-time PCR and melting temperature (T_m) analysis

Specific melting temperature (T_m) is fundamental characteristic of DNA; it is a function of GC/AT ratio, nucleotide sequence, and DNA length [170,171]. In regard to these characteristics and RT-PCR instrument-based automatization, many researchers tried to find sufficient sequence variation (Tm) of particular amplified gene (or DNA sequence) to differentiate *Borrelia* species. Genes *hbb, p66, recA, ospA*, and *groEL* were employed more or less successfully; species differentiation is of special interest in Europe where more than one *Borrelia* species is present [61–63]. Portnoi et al. and Ferdin et al. differentiated *Borrelia* species based on *hbb* gene which enables distinction between majority of *Borrelia* species although it fails to discriminate between *B. spielmanii* and *B. valaisiana* [48,61]. Many laboratories are trying to introduce the method in clinical diagnostics and enzootic studies because of its simple handling and possible species yield; however, it was not found to be optimal for clinical samples.

3.4. MLST analysis

MLST uses chromosomally located housekeeping genes that evolve slowly. The advantage of the method is a high

discriminatory power, although it is time consuming and costly. This highly sensitive typing method is a valuable tool for the population studies, phylogenetic analysis, epidemiological monitoring, and evolutionary studies [64,65]. Generally, the method requires amplification and sequencing of several housekeeping genes, in *B. burgdorferi* sensu lato eight genes are included, *clpA* (Clp protease subunit A), *clpX* (Clp protease subunit X), *nifS* (Aminotransferase), *pepX* (Dipeptidylaminopeptidase), *pyrG* (CTP synthase), *recG* (DNA recombinase), *rplB* (50 S ribosomal protein), and *uvrA* (Exonuclease ABC) [158]. Borrelia MLST scheme is available through the MLST network (http://www.mlst.net/) [64,65,158]. MLST method enabled separation of *B. bavariensis* from *B. garinii* together with epidemiological data (birds were determined as reservoir of *B. garinii* while small mammals of *B. bavariensis*) and separation of *B. finlandensis* from most closely related *B. burgdorferi* sensu stricto [1]. The method is not appropriate when dealing with mixed samples, since it is virtually impossible to determine the correct sequences. It is not necessary that mixed infection will be apparent in all loci, but if present even in one locus, the entire sample should be omitted from further analyses [66].

4. Molecular methods for detecting borrelia in host reservoirs and vectors

PCR-based molecular techniques represent sensitive and specific methods for detection, identification, and genotyping of borrelia not only in ticks but also in reservoir hosts and experimentally infected animals [67]. The main aim of molecular genotyping is to assess relationship between *Borrelia* species (and/or subtype) and its reservoir hosts and vectors as well as to clarify molecular background of pathogenicity.

Confirmation of infection in reservoir host and ticks can be achieved by culture or PCR detection of borrelial DNA, in reservoir host main samples are ear, heart, lung, or urinary bladder biopsy [68–71].

A number of the previously described molecular approaches have been used for analyzing borrelia in ticks and reservoir hosts. OspC typing was applied to investigate B. *burgdorferi* sensu lato genetic diversity in environmental samples [47,72,73,166], variation of *rrfA-rrlB* and *rrs-rrlA* was employed for genotyping of tick originated samples [66] and MLST was used for genotyping borrelia from questing ticks and rodents [69,74,75].

Molecular methods also enable detection of the infections by multiple *B. burgdorferi* sensu lato species in contrast to cultivation, where one species generally overgrow the other [68]. High incidence of mixed borrelia infection in ticks has been reported [74,76].

5. Expert commentary

Typical erythema migrans is usually sufficiently distinctive to allow a clinical diagnosis of Lyme borreliosis, for all other clinical presentations the diagnosis should rely on laboratory confirmation. The majority of laboratories perform tests based on the detection of specific borrelial antibodies in the serum. Major disadvantage of serology represent low sensitivity in early manifestations. On the other hand, molecular methods enable direct detection of the agent before the appearance of specific antibodies. Nevertheless, PCR testing has limitations as well, it does not allow distinction between living and dead organisms, and there is a lack of standardization in the sample preparation, target genes, detection methods. Diagnostic potential of PCR differs regarding to clinical presentations. Sensitivity of PCR in skin biopsies from EM patients is usually high but PCR together with culture and serology is primarily used in research studies, since the diagnosis is made on the basis of history and visual inspection of the skin lesion. Diagnostic approach in the case of ACA includes clinical features together with histologic findings and serology, which is almost always positive; PCR may serve for supporting diagnosis but is mostly used in research studies. Laboratory diagnosis of Lyme neuroborreliosis is based on pleocytosis and demonstration of intrathecal synthesis of specific borrelial antibodies; molecular tests can support diagnosis but their main limitation is low diagnostic sensitivity. In Lyme arthritis, PCR can be used as supporting diagnostic testing. For all clinical features, PCR tests designed not only to confirm infection but also to determine *Borrelia* species and subtype under the species direct in clinical samples are preferred. Moreover, molecular methods try to clarify background of very complex *Borrelia*–host interaction. Beside the human pathology, molecular methods are very often utilized in epidemiological, and evolutionary studies for detection, identification and genotyping of *Borrelia* species in ticks, reservoir hosts and experimentally infected animals.

Molecular methods are promising and currently serve as supporting diagnostic testing in Lyme borreliosis. Since the field of molecular diagnostics is under rapid development, molecular testing could become an important diagnostic modality.

6. Five-year view

Molecular methods currently serve as supporting diagnostic testing in Lyme borreliosis. The field of molecular diagnostics is under rapid development and molecular testing could become an important for diagnostics. Since diagnostic sensitivity of molecular methods is one of the main limitations, novel methods enabling enrichment of microbial DNA in the sample could offer a solution.

The main development in the field of molecular methods could be on the site of NGS technologies, which are already a routine part of biological research. With evolution of NGS technologies and reduced costs, it is anticipated that NGS analysis will also be used for genotyping.

Serology will remain an important diagnostic method and will be performed in majority of diagnostic laboratories. There is already a great deal of automatization in enzyme and chemiluminescence immunoassay and it is expected that further automatization will be also in the field of immunoblot assays.

Key issues

- Laboratory confirmation of infection is required for all stages of the infection, except for erythema migrans
- Serologic methods are recommended as primary diagnostic testing in the Lyme borreliosis

- Limitations of serology are low sensitivity in early infection, cross-reactivity, inability to distinguish active from inactive infection
- Direct detection of the pathogen would be needed, culture is too insensitive.
- Molecular methods currently serve as supporting diagnostic testing in Lyme borreliosis. Since the field of molecular diagnostics is under rapid development, molecular testing could become an important diagnostic modality.
- Main limitations of molecular methods are low diagnostic sensitivity and lack of standardization in the sample preparation, target genes, detection methods
- Molecular methods are utilized in epidemiological, clinical, and evolutionary studies for *Borrelia burgdorferi* sensu lato genotyping
- Molecular methods play an important role in detection, identification and genotyping of *Borrelia* species in ticks, reservoir hosts and experimentally infected animals.

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Declaration of interest

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