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Nucleic Acid Amplification Based Diagnostic of Lyme (Neuro-)borreliosis – Lost in the Jungle of Methods, Targets, and Assays?

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Abstract: Laboratory based diagnosis of infectious diseases usually relies on culture of the disease causing micro-organism, followed by identification and susceptibility testing. Since *Borrelia burgdorferi sensu lato*, the etiologic agent of Lyme disease or Lyme borreliosis, requires very specific culture conditions (e.g. specific liquid media, long term culture) traditional bacteriology is often not done on a routine basis. Instead, confirmation of the clinical diagnosis needs either indirect techniques (like serology or measurement of cellular activity in the presence of antigens) or direct but culture independent techniques, like microscopy or nucleic acid amplification techniques (NAT), with polymerase chain reaction (PCR) being the most frequently applied NAT method in routine laboratories.

NAT uses nucleic acids of the disease causing micro-organism as template for amplification, isolated from various sources of clinical specimens. Although the underlying principle, adoption of the enzymatic process running during DNA duplication prior to prokaryotic cell division, is comparatively easy, a couple of 'pitfalls' is associated with the technique itself as well as with interpretation of the results.

At present, no commercial, CE-marked and sufficiently validated PCR assay is available. A number of homebrew assays have been published, which are different in terms of target (i.e. the gene targeted by the amplification primers), method (nested PCR, PCR followed by hybridization, real-time PCR) and validation criteria. Inhibitory compounds may lead to false negative results, if no appropriate internal control is included. Carry-over of amplicons, insufficient handling and workflow and/or insufficiently validated targets/primers may result in false positive results. Different targets may yield different analytical sensitivity, depending, among other factors, of the redundancy of a target gene in the genome. Performance characteristics (e.g. analytical sensitivity and specificity, clinical sensitivity and specificity, reproducibility, etc.) are, if available, only applicable to a specific assay, running in a specific laboratory. Finally, not only the NAT/PCR method itself, but also the process of DNA isolation from the specimen, is highly diverse and may have fundamental impact on the (expected) PCR result. Of concern are distribution effects of DNA, in particular, if only low numbers of bacteria/genomes are present in a sample, as it is the case for instance in cerebrospinal fluids.

For the ordering physician and for the patient requesting PCR analysis, these 'pitfalls' are usually invisible. As a consequence, the reported result (i.e. PCR negative or positive for *B. burgdorferi*) is hard to interpret, especially, if the reported PCR result is contradictory to the clinical diagnosis or other laboratory findings. Moreover, due to the high number of different assays in use, two laboratories, testing the same specimen, might come to different PCR results.

The current paper wants to summarize the available PCR/NAT assays for the detection of *B. burgdorferi* DNA in clinical specimens, with special attention to neurologic disorders, and to discuss the difficulties in PCR analysis and result interpretation, associated thereof. In view of growing numbers of patients who are diagnosed of having Lyme disease, and acknowledging a substantial growth in knowledge regarding other tick- or vector-borne pathogens, which might be able to induce symptoms comparable to Lyme (neuro-)borreliosis, efforts are urgently needed to standardize and harmonize methods for *B. burgdorferi* nucleic acid amplification.

Keywords: Polymerase chain reaction, Borrelia burgdorferi, Lyme disease, Lyme neuroborreliosis.

INTRODUCTION

The invention of polymerase chain reaction has revolutionized human diagnostics in many fields and in many ways. In infectious diseases, the confirmation of the presence of slow growing microbes like *Mycobacterium tuberculosis* dropped from 4 - 8 weeks (culture) to less than one day (modern real-time PCR assays). Especially in diseases, which are caused by uncultivable pathogens (e.g. Syphilis, caused by the spirochete *Treponema pallidum* or gastrointestinal disease caused by *Tropheryma whipplei*), the molecular detection of pathogen DNA by amplification is of great importance. PCR – or in general, **n**ucleic acid **a**mplification

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techniques, NAT or NAAT – protocols have been developed for a wide variety of pathogenic micro-organisms, and many of these protocols have taken their way into routine diagnostics. Moreover, NATs contribute to unraveling the nature of novel, emerging diseases, as was the case with the very recently identified, tick-transmitted SFTS (severe fever with thrombocytopenia syndrome) virus [1, 2]. Since there is no doubt about the potential and impact of NAT on medicine and, in particular, diagnostics, a couple of obstacles and problems are stunningly still associated with DNA/RNA amplification. The current paper aims to provide a methodological overview and critical discussion about the power, the potentials, limitations and open questions of PCR with special attention to PCR-based diagnosis in Lyme disease patients.

POLYMERASE CHAIN REACTION, SOME BASIC REFLECTIONS

The underlying principle of PCR is comparatively simple. Mechanisms, which are well known from the DNA duplication prior to prokaryotic cell division, are adapted to synthesize novel DNA molecules *in vitro*. While during replication of DNA *in vivo*, the DNA polymerase needs a short ribonucleic acid oligonucleotide to start with, provided by the enzyme primase (reviewed in [3]), during PCR *in vitro* these starter molecules are synthetic (the oligonucleotide primers). These primers define the starting points for DNA synthesis anywhere on the bacterial genome, regardless of an origin of replication. The use of at least two, freely designable primers is the true power of the invention, since instead of linear duplication logarithmic increase of target molecules is enabled. As a consequence, the extremely low amounts of DNA present in clinical samples and invisible with routine diagnostic devices are pushed beyond the border of visibility, making them detectable by standard gel electrophoresis or by detection of fluorescence, which is emitted during real-time PCR.

With the advent of thermo stable DNA polymerase, a cyclic temperature profile for

i) melting the DNA double strand at 95 C (the original template as well as the newly synthesized amplicons),

ii) enabling annealing of the primers at low temperatures (usually between 50 and 60 C) and

iii) allowing polymerase to elongate from the primers at ambient (72 C) temperatures, was possible, allowing access of PCR into medical diagnostics [4, 5].

Meanwhile, important modifications of the original protocol have been developed (Table **1**) and the whole process of DNA extraction and amplification in a real-time fashion is highly automated.

Following a steep increase in newly developed PCR applications, published the years after first description, some serious limitations and obstacles became clear. Among those were difficulties, associated with the design of primers and the development of reliable PCR protocols, which are of interest for the technically interested user. A by no means complete collection of critical issues and parameters is provided in Table **2**. In case, FDA (US Food and Drug Administration) cleared or IVD-CE (*In Vitro* Diagnostics Community European) marked assays are used as test format, the

Table 1. Overview Different PCR Formats/Assays

Assay Format	Oligonucleotides	Analysis	Rating
classical PCR format	2 primers	electrophoresis, size of band	not appropriate for diagnostics, comparatively low analytical sensitivity, high risk of false positive results due to lack of specificity confirmation (band size not indicative for the expected amplicon!), acceptable, if downstream analysis (e.g. hybridization [see next row] or sequencing is done)
PCR/hybridization	2 primers, one probe	electrophoresis and subsequent blotting, novel formats use reverse blotting (probe immobilized on blotting membrane or solid support)	specificity of the expected band is confirmed by probe hybridization, hybridization signal enhances sensitivity
nested PCR	2 primers first round, 2 internal primers second round	gel electrophoresis, size of band	enhanced specificity due to internal primer pairs which act as probes, high risk of contamination (carry over) when opening the tubes of the first round, high analytical sensitivity
real-time PCR	2 primers, fluorescent intercalating dye (e.g. Cybergreen)	analysis of fluorescence, emitted during DNA-synthesis ("real-time"), no confirmation of band size	enhanced analytical sensitivity due to fluorescence, low contamination risk as being carried out in closed reaction vials, no need to open vials after PCR is finished, low specificity (staining of any double stranded DNA molecule), analysis of melting curves for increase in specificity
real-time PCR	2 primers, 1 or 2 probes (depending on the actual format)	analysis of fluorescence, emitted during DNA-synthesis ("real-time"), no confirmation of band size	high analytical sensitivity due to fluorescence, low contamination risk as being carried out in closed reaction vials, high specificity

office physician/general practitioner or the treating physician in a hospital setting does not necessarily need to know about such technical details, since the most critical variables are usually solved by the manufacturer prior to clearance or during routine perfective maintenance. There are, however, things which are important to know to correctly rate a "positive" or a "negative" on the laboratories' report in case, home-brew assays (i.e. laboratory developed and validated PCR formats) are used, as is the case for most if not all *Borrelia* PCR applications. In the following, some critical aspects of "Lyme Borrelia complex" PCRs are discussed.

APPLICATIONS AND TARGET GENES

Very early after introduction of NAT into medicine, first protocols for the detection of DNA of *Borrelia burgdorferi* were published, e.g. a culture based PCR [6], a PCR for detection of *Borrelia* DNA in erythema migrans [7] or for the diagnosis of Lyme neuroborreliosis [8]. Since *B. burgdorferi sensu lato*, the etiologic agent of Lyme borreliosis (LB, European term) or Lyme disease (LD, US term) is difficult to cultivate, PCR for the detection of *Borrelia* DNA became a favorite technique in the early 90's of the 20th century. Since that time, a couple of different assays have been published, although, unlike for other indications, a commercial, IVD-CE-marked/FDA-cleared and sufficiently validated assay is still not available. A couple of different targets have been addressed by primer/probe combinations, for instance the 16S-gene [9], the *rrf-rrl* intergenic region (also knonw as 5S-23S intergenic spacer region) [10, 11], the flagellin gene [12], p66 outer membrane gene [13], the plasmid located *ospA* gene [7], or *Ly*-1 (*rpoC*) [14]. As a consequence, different assays, mostly in house protocols, are in use throughout the laboratories. This lack of standardization is not only an academic problem but actually contributes to the difficulties in diagnosing LB/LD and to define clear parameters for case definition based on the direct detection of the pathogen.

"HOME-BREW-ASSAYS"

In the early days of PCR, individual, home-brew protocols were used (i.e. each laboratory performed its own in house tests with its own primer pairs and protocols) but the number of available test components (i.e. consumables, extraction chemistry, amplification chemistry like nucleotides, polymerases and primers) was limited. With the increasing use of NAAT tests applied in infectious disease diagnostics, two developments have run in parallel: more and more companies provided consumables, enabling researchers and diagnosticians to tailor specific applications, and the increased workload due to increased use of NAAT in diagnostics forced laboratory staff and companies to think about methodological standardization and automation in order to allow for medium and high throughput diagnostics. A previously laborious procedure, the extraction of the nucleic acids from the specimen, was transferred from manual isolation (i.e. phenol/chloroform extraction and ethanol precipitation) to more standardized extraction over silica columns (i.e. for each patient the same amount or volume of specimen was extracted in always the same matter). Soon, the researcher or diagnostician had to choose from a plethora of different assays, available [15]. Following the increasing use of PCR in diagnostics, manufactures started to automate DNA extraction. Nowadays, two dozen or more instruments are on the market, allowing for (semi-)automated nucleic acid extractions from divers starting material and in each case with divers extraction kits for specific applications. This means that neither extraction nor amplification is really standardized. A high degree of standardization and comparability is only achieved when using IVD-CE-marked or FDA cleared assays for the detection. This is, for instance, the case with virus load determination in HIV diagnostics [16] for which highly standardized extraction and amplification protocols from two main manufacturers are available. For Lyme borreliosis diagnostics, however, no such assay is currently on hand, and published results of home-brew-assays are hard to compare. This may be exemplified by two PCR investigations, done 10 years apart from each other. GOOSKENS *et al.* (2006) [17] detected *Borrelia* DNA in 50% of CSF-samples with pleocytosis but only in one CSF out of 15 (7%), obtained from patients without pleocytosis but with neurological symptoms of Lyme disease and positive serology (PCR: *ospA* PCR, 500 µL CSF, 100 µL elution, real-time assay). Six of 16 (38%) CSF from patients with acute Lyme neuroborreliosis (14/16 with pleocytosis) but 25% of 44 CSF of patients with chronic neuroborreliosis yielded amplification with one of two slightly different *ospA* PCR's (PCR: 100 µL CSF, 30 µL elution, hybridization with radiolabeled probe) in the study of NOCTON *et al.* (1996) [18]. One reason, among others, for the different sensitivity of both PCR might be the primer pair chosen, with the older primer pair [18] being the less specific one, as shown later in this paper.

LYME BORRELIOSIS/LYME DISEASE

Lyme borreliosis is without any doubt the most frequent bacterial disease, transmitted by an arthropod vector in Europe. However, since the disease is not always manifested with its characteristic symptoms, the actual incidence, even in countries were a compulsory registration exists, remains largely unclear. Improvements in diagnostics could contribute to better mapping the actual incidence of the disease. However, a general increase in the number of reported cases is obvious, as it is the case for the geographic area, in which LB is endemic [19]. While this increase might be a consequence of higher awareness, a true change in epidemiology cannot be ruled out.

The complex background of the multiorgan infectious disease LB is discussed elsewhere in this supplement. It should be noted, however, that beside some characteristic clinical manifestations and a couple of clear laboratory parameters, many patients suffer from more or less non-specific symptoms, making a clinical, i.e. symptom based diagnosis difficult. In some cases, definite diagnosis of (neuro-)borreliosis is further hampered by a lack of indicative laboratory findings (e.g. lack of specific antibodies in serum and/or CSF, absence of pleocytosis). This has caused debate, what is an accepted case of (neuro-)borreliosis and what is more likely another infectious disease. A problem regularly encountered is the question whether a patient suffers (neuo-)borreliosis or if a different diagnosis is more likely, including the conclusion that the actual symptoms being observed might be the result of a non-infectious, per-

haps mentally driven disorder. Enabling general practitioners and hospital physicians to draw the correct conclusion and the exact diagnosis for the patient is the challenge of laboratory medicine.

In case of infection or disease, *B. burgdorferi sensu lato* can be found in virtually any organ and any part of the human body, depending on the actual manifestation of Lyme disease. However, the amount of detectable bacteria in a given compartment of the body may be very low, and sometimes even too low to be actually detected by PCR.

Borrelia Burgdorferi Sensu Lato

The genus *Borrelia* can be divided into the relapsing fever group and the borreliosis group. SATZ (2010) [20] lists twelve *Borrelia* species as belonging to the borreliosis group (commonly referred to as *B. burgdorferi senus lato*) and an additional three species, causing borreliosis in animals, only. Two of these (geno-)species, *Borrelia bissettii* and *Borrelia andersonii*, are not listed in the "list of prokaryotic names with standing in nomenclature" (LPSN) [21], while an additional species, *Borrelia bavariensis*, proposed recently [22] is neither mentioned in [20] nor in the LPSN [21] as being an accepted species. Newly described and mentioned in the LPSN are *Borrelia americana* and *B. carolinensis* [23, 24] which are also listed in a current review [25], which includes 18 species in the "Lyme *Borrelia* complex".

Although the *B. burgdorferi senu lato* complex is large in terms of the number of described genospecies, only a few of them are indeed associated with LB/LD. These are the classical three genospecies *Borrelia burgdorferi sensu stricto*, *Borrelia garinii*, and *Borrelia afzelii*. In recent years it became evident, that a fourth species, *Borrelia spielmanii*, is causing disease, too. Three further species have been found in single LB cases, namely *Borrelia valaisiana*, *B. bissettii* and the recently described *B. bavariensis*. The taxonomic status of the latter, i.e. whether being a true species [22] or only a subtype of *B. garinii* is still subject of debate.

As a consequence, primers or primer/probe systems for amplification of borrelial DNA need to be specific for the relevant *Borrelia* species, or, if not, further characterization of amplicons is required (i.e. by sequencing). This may exemplified by the widely distributed "tick PCR" (a PCR which is applied to tick extracts to screen whether a tick was positive for *Borrelia* or not). Use of broad reactive primers like 5S – 23S rDNA intergenic spacer specific primers [10, 11] may pose the risk of leading to positive results even in the presence of non–pathogenic *Borrelia* or *Borrelia* for which the actual pathogenicity or degree of virulence is not known. This has been shown in our lab, when we demonstrated that by using the mentioned primer system DNA of the *Borrelia* LB2001 complex (related to a group of non-virulent/non-pathogenic relapsing fever spirochetes) could successfully amplified from ticks, removed from humans in the South-Western parts of Germany (unpublished results). On the contrary, PCR assays targeting conserved regions only available in the classical three *Borrelia* genospecies are prone to fail amplification in cases, in which one of the newly described pathogenic *Borrelia* is associated with disease.

PRIMER SEQUENCES

Many of the available PCR protocols have been published a couple of years ago, sometimes soon after introduction of PCR into routine diagnostics. Since the primers define the specificity of the PCR they need to be designed and validated with care to avoid non-specific binding, leading to false positive or false negative PCR results [26, 27]. For the current paper, two primer pairs, one for a genomic and one for a plasmid encoded target were chosen and checked for specificity by using the NCBI BLAST tool (available online: http://blast.ncbi.nlm.nih.gov/Blast.cgi; option nucleotide blast), by BLASTing the primer sequences against the nucleotide collection (nr/nt), excluding models (XM/XP) and uncultured and/or environmental sample sequences.

The *Ly*-1 primers (which actually bind to *rpoC*, a subunit of RNA polymerase and thus a genomic target [14]) were found to be specific for *B. burgdorferi*, *B. afzelii*, *B. garinii*, thus still being useful primers for amplification. One of the primers, specific for the plasmid encoded *ospA* (OspA18-39) [28], binds in silico to the genus *Borrelia*, but also *Bacillus thuringiensis* genome, *Arabidopsis thaliana*, *Vitis vinifera*, *Medicago truncatula*, the human chromosome 14, but shows reduced sequence homology (i.e. a couple of mismatches) even to a large number of *Borrelia* sp. isolates. Since further conditions like $MgCl_2$ concentration, primer concentration, annealing temperature and even the type of polymerase used may influence and contribute to the specificity of a PCR (Table **2**), a less specific primer like OspA18-39 poses the risk of non-specific amplification. Interestingly, two cases of false positive *Borrelia* PCR discussed in the literature [26, 27] both could be linked to *ospA*-PCRs. Although the reason for the misleading results is not clear, primers must not only be screened for specificity during development of an assay but also on a regular basis (e.g. annually), to avoid false positives.

Actually, the problem of older primer sequences is that the primers were designed and validated at that time on the basis of a very few, some from nowadays perspective even bad, sequences. Consequently, before using these primers in an own assay the oligonucleotide sequences should be checked carefully against available sequences. One of the *ospA*-primers published in 1991 [7] for instance aligns perfectly with some American *Borrelia ospA* sequences and some *B. valaisiana* sequences. To the majority of *ospA* sequences available today, however, these primers won't fit. In order to check for the accuracy of *ospA*-primers, a couple of published primer were aligned with the MEGA 5.0 software [29] to 43 publicly available *ospA*-sequences from *B. burgdorferi* s.s. (13), *B. afzelii* (10), *B garinii* (1), *B. valaisiana* (11), *B. spielmanii* (3), *B. japonica* (4) and *Borrelia* species (1) and checked for the number of mismatches (Table **3**). Only the most recent primer pair used by GOOSKENS *et al* (2006) [17] was found able to detect virtually all know *Borrelia ospA* sequences, using this *in silico* approach. Interestingly, the reported PCR assay showed a comparatively high sensitivity in CSF specimens with pleocytosis, although rather low volumes (500 µL) were used for extraction.

Table 2. Parameters Which have Significant Effects on the Performance of PCR Reactions, with Special Emphasis on *Borrelia* PCRs. The Table Mostly Reflects the Authors Experience, Some Aspects are in Addition Cited from Other Publications [42, 53, 54, 55]

Parameter	Critical Issues
sample volume	• definition of a minimum volume required to meet the minimum amount of genomes required for reproducible amplification results, • for samples with low cfu load, 1 mL should be the minimum amount of sample to start with
sample type	although DNA can be extracted from virtually any clinical specimen, some limitations exist: • tissue samples fixed with formaldehyde are generally less favorable than native specimens, • the specimen should be taken from a site where an infection is likely
extraction method	• extraction kits or methods of different suppliers may not be comparable, DNA extracted with one procedure might lead to optimal amplification in a given PCR while an extract of the same specimen but extracted with a different procedure might fail to yield amplification
target	• target must be sufficiently conserved to allow amplification of every isolate of the clinically relevant species but should be sufficiently low conserved to allow for discrimination between clinically relevant and irrelevant species • redundant targets may be of advantage (although the analytical sensitivity can't be less than one genome per PCR) • targets which are encoded on genomic DNA seem to be superior over plasmid encoded targets since the faith of plasmids following cell death is even less clear than for genomic DNA • a mRNA based target offers the opportunity to discriminate between "DNAemia" (simple presence of DNA) and true colonization/infection
template DNA	• inhibitors may be present even after extraction with commercial kits (for instance due to an excess amount of eukaryotic DNA, which might lead to inhibition of PCR), the use of wrong containers may also lead to inhibition (heparinized blood has a higher risk of causing subsequent inhibition than citrated blood or blood with EDTA as anticoagulant) , • the volume of template used for amplification is critical: if low numbers of target DNA are expected, volumes of 10 µl up to 30 µL of template DNA may be advisable
primers	• primers are the most critical components of PCR applications since they define specificity; • annealing characteristics greatly influence sensitivity (lower annealing temperature increase non-specific binding while higher temperatures impede binding even to the matching target sequence), • annealing is influenced not only by the annealing temperature but also by the chemistry of the PCR reaction (e.g. the primer concentration itself, the concentration/amount of template DNA present, $MgCl_2$ concentration, presence/absence of glycerol or other components); • mismatches in primer sequence compared to target sequence may lead to reduced analytical sensitivity and specificity (mismatches in the central part of primers may show moderate effects, 5' mismatches may be without effect, depending on the length of the primer whereas 3' mismatches can be detrimental (the correctly positioned 3' end with its free 3' hydroxygroup is essential for elongation by DNA polymerases), • primers may form hairpins, dimers or multimers, depending on the sequence and the PCR-conditions, • multiple primers as in multiplex PCR's may reduce analytical sensitivity; • primer sequences should be checked in silico (BLASTing against nucleic acid sequence databases) on a regular basis to ensure specificity (newly published sequences may not be targeted by primers due to genetic variations or novel strains/variants detected; also, newly published sequences of, for instance, saprophytic bacteria might cause concern, that cross-reaction might be possible under certain circumstances; primer sequences should be than adapted accordingly and the PCR needs revalidation!)
PCR chemistry	• in addition to $MgCl_2$ a couple of components have influence on the amplification efficacy; some additives may augment amplification while other may increase specifity.

PLASMIDS OF BORRELIA

The genus *Borrelia* is belonging phylogentically to the Gram-negative bacteria and here into the order Spirochetales. Close relative is the Genus *Treponema* with the syphilis causing spirochete *T. pallidum*. At this time, a couple of whole genomes of *B. burgdorferi sensu stricto* as well as genomes of *B. garinii* and *B. afzelii* have been sequenced with the genome sequence data being available in public databases. It is likely that in the near future more genomes will become available, in particular for those *Borrelia* species which are currently not sequenced. The genomes of the sequenced *B. burgdorferi* are comparatively small, being less than 1 million bp in size. However, some genospecies of the complex have accumulated extra genetic material in the form

of up to twelve linear and nine circular plasmids, which can account for up to another 600.000 bp, a phenomenon nearly unprecedented in the bacterial kingdom [30].

TARGET IMBALANCE

Some plasmids may be single copy plasmids while other plasmids may be present in more than copy per *Borrelia* cell. Consequently, a plasmid target based PCR would have a higher chance of yielding a positive result than a single copy gene, located on the bacterial chromosome. This was shown as early as 1994 [9] and was named 'target imbalance'. In culture negative clinical specimens of 19 patients, plasmid encoded targets (*ospA*/*ospB*) were detected by PCR in each case, while PCR for the 16S target yielded amplification in eight, and PCR targeting the flagellin gene in nine cases (eleven specimens positive in one of both PCRs). The authors concluded that "the most sensitive and reliable targets for PCR detection of *B. burgdorferi* lie on extrachromosomal elements" [9] although they acknowledged that plasmids may be released in membrane vesicles released by *Borrelia* [31].

PLASMIDS AND GENOME PLASTICITY

Many of the plasmids occurring in species of the "Lyme complex *Borrelia*" are already sequenced. Nevertheless, novel variants of plasmids are described in the most recent literature as was the case for a large linear plasmid of *B. spielmanii* [32], a genospecies which appears to be associated with skin manifestations [33]. At least during prolonged culture and repeated passage, *Borrelia* might even loose plasmids [34], illustrating both, the high genetic diversity of the complex as well as the urgent need for more research in order to get a more detailed and comprehensive view about the genetic material and genomic plasticity of the *B. burgdorferi* complex.

PCR assays have been described for both, plasmid coded targets and targets located on the borrelial chromosome. Plasmids in general constitute additional genetic material and a given bacterial species may acquire plasmid DNA from external sources or *via* direct transmission from a donor bacterium. Hence, many plasmids are mobile and may easily be transferred between strains of a particular species. Some plasmids, however, are even more promiscuous, as is the case with the blaNDM$_{-1}$, conferring the ß-lactam resistance of New Delhi metallo-ß-lactamase-1 type. NDM-1 was first reported from a *Klebsiella pneumoniae* but has spread into virtually all members of enterobacteriaceae and the nonfermenter within only three years, illustrating the capacity to not only cross a species- but also a genus-border (discussed in [35]).

For the *ospC* coding plasmid transferability by means of lateral gene transfer within the *B. burgdorferi* complex has been shown [36], although the exact mechanism, by which genetic material is exchanged, remains unknown. Since plasmid containing membrane blebs are released by *Borrelia* [31, 37] a potential vehicle for genetic exchange might already be identified. If, however, for instance the *ospA* carrying plasmid of *B. burgdoferi* would be transferable, this would have great impact on the interpretation of PCR results.

Associated with the question of plasmid mobility is the possible persistence of plasmid DNA in tissue or body fluids. Once a *Borrelia* infection is successfully fought by the immune system, the cellular debris is cleared from the body. Many authors have shown, that during the course of an infection, *B. burgdorferi* PCR from urine is positive [38-41]. Genomic DNA, however, can be detected even after clearance of infection. Li *et al.* (2011) [42] reported in patients with antibiotic-refractory arthritis persistent genomic DNA causing positive PCR for up to 11 month. The authors used a smart approach in which they not only looked for genomic DNA but also for (instable) mRNA, which can be assumed to be present only during active borreliosis (see below). At least at this time, persistence of plasmids after clearance of *Borrelia* infection cannot be excluded with sufficient reliability. This would probably mean that even in cases in which *Borrelia* infections may not have caused disease, a plasmid-based PCR (i.e. a PCR targeting *ospA* or *ospB*) would stay positive for an unknown period of time.

DNA VS. mRNA

The persistence of DNA (either as 'dead' microorganisms in tissue or phagocytic cells, as DNA remnants in tissues or fluids, or as DNA containing membrane vesicle) raises a couple of questions. What is the actual relevance of detected DNA by means of a positive PCR? Is a positive amplification a true or reliable surrogate for active disease/infection? What, if a PCR signal is only a result of remnant DNA, comparable to what is known as DNAemia in molecular sepsis diagnostics [43] and how can 'dead DNA' be discriminated from vital or at least living microorganisms? Recently, Li *et al.* (2011) [42] reported that in eight of eleven samples from erythema migrans not only *Borrelia* DNA was amplified but also *Borrelia* specific messenger RNA (mRNA). In eleven samples from synovial fluids, however, no mRNA was detected. While DNA is comparatively stable, mRNA is a transient molecule with short half-life, synthesized more or less continuously by RNA-Polymerase during protein biosynthesis [44]. Consequently, the detection of this molecule rules out the presence of remnant DNA ("DNAemia"). This approach is truly worth to be further investigated. Although laborious and not suitable for routine application at this time, (co-)amplification of mRNA would be a true surrogate marker for active infection. Whether an infection is the reason for the observed symptoms or disease remains a question of clinical diagnostics in anyway.

DNA EXTRACTION

The extraction of nucleic acids from the clinical specimen is one of the most critical issues, since the amount and the quality of target DNA yielded determines the outcome of the PCR process. Target DNA may be lost during extraction or poor extraction procedures may yield DNA 'contaminated' with inhibitory components. Although standardized procedures are available, e.g. the column based technologies, the magnetic bead based separation and others there is indeed a lack of standardization in the procedures for nucleic acid isolation. A recent market analysis of the 'Laborjournal', a German lab magazine, revealed more than 40 differ-

Table 3. Specificity of Published *ospA* Primers. *OspA* Specific Primers Available from the Literature (Reference in Column 1) were Aligned Against 43 *ospA* Sequences of Different *Borrelia* Species, Using MEGA 5.0 Software (TAMURA *et al* 2011). Bold Faced Upper-case Letters (A/T/C/G) Indicate that the Particular Base Matches to All 43 Aligned Sequences While Lower Case Standard Letters (a/t/C/g) Indicate that There is a Mismatch with at Least one Sequence of the Alignment; r/R Means Degenerated Base Position C or T, the Elongation Direction is Indicted by ► or ◄. The Primer Sequences were Taken from the References but Adjusted (i.e. Reversed) where Necessary to Fit for the MEGA Alignment. Primer Names as Given in Column 2 are Taken from the Original Publications. In the Last Column, a Comment is Which Provides Additional Information about the Specificity, i.e. if A primer Pair was Designed Specifically for a Subset of *Borrelia* Isolates

Authors [Reference]	Primer Names	Primer Names/Sequences	Comment
Guy & Stanek 1991 [7]	primer pair 1 N1: C1: primer pair 2 N2: C2:	 GAgcTtAAAGGAACTTCTGATAA► ◄ACAATTACagTacAAcaaTAc AtGGaTCtGGagtaCTtGAA► ◄AgAaGgaactgtTacTttaag	The primer/probe system is adapted to American *B. burgdorferi* isolates, to which all oligonucleotides show 100% identity.
Demaerschalck *et al.* [56]	OspA_fw: OspA_rw:	aATAGGTcTAaTAatAGCCTTAATAGC► ◄ tTtTcAAAGAAGATGgcaaAACAc-TAG	The primers are specific for the three 'classical' genospecies, when aligned only to them the number of mismatches is 2 for OspA_fw and 4 for OspA_rw.
Gooskens *et al.* [17]	BORs: BORas:B OR-TQ (probe):	ATATTTATTGGGaATAGGTcTAaTAT► ◄CTTGTAAGrAAAGAAAAagAcAAaG AAgCAAAATGTTAGCagcCTtGA	
Nocton *et al.* [57]	OspA4: OspA2: OspA3(probe):	CtgcagctTGGaattcaggcacTtc► Ggtcagcagttgaaattacaaaac CagTacAAcaaTAcgACtCaaatGgc	The primer/probe system is adapted to American *B. burgdorferi* isolates, A3 and A4 show 100% homology, while A2 has two mismatches. The shown primer/probe system is Set 1 in the original publication of 1994
Nocton *et al.* [57]	OspA149: OspA319: OspA6'(probe): OspA6 (2nd probe):	ATGAAAAAATATTTATTGGGaAT► ◄ ACAGTAGACAAgcTTGAgcTtAAAG GCATGtAAgCAAAATGTTAGC ATTGGGaATAGGTcTAaTATtAGCcT	The primer/probe system is also adapted to American *B. burgdorferi* isolates, A319 has one mismatch, A6 two while A149 and A6' show 100% homology. Primer/probe system OspA149/319/6' is Set 2 in the original publication of 1994, while OspA149/319/6 is Set 3
Priem *et al.* ("ospA"-primers) [28]	outer primer 1: outer primer 2: nested primer 1: nested primer 2:	GGGaATAGGTcTAATATTAGCc► ◄ acTtccACttTAACaaTTagTg gCAAAATGTTAGCagcCTtGAt► ◄ GGAACcAgACTTGAATAcaCAg	The primer/probe system is also adapted to American *B. burgdorferi* isolates, all but nested 2 show 100% homology, nested 2 displays 2 mismatches.
Priem *et al.* ("ospA/B"-primers) [28]	outer primer 1: outer primer 2: nested primer 1: nested primer 2:	TTGTAAgCAAAGAAAAaaA► ◄ ttaaaaacGCTTTaAAATAA* GAcGgcAAgTACgatCTAgctG► ◄ ttaaAgAaGgaactgtaacT	The primer/probe system is also adapted to American *B. burgdorferi* isolates, primer 1 fits 100%, nested 1 shows 3 mismatches, nested 2 one mismatch and primer 2 fits 100% is strain Fort Sheridan 36 is omitted. * Outer primer 2 is shown reversed in the original paper.

ent providers, offering more than 250 kits for RNA extraction, covering virtually every source of possible specimen [15]. Although no market analysis for DNA extraction kits was done, one can assume that the spectrum is comparable.

Most suppliers of commercial kits have focused on adapting the extraction procedure to modern automated solutions. Depending on the extraction kit used and the automat available, volumes between 200 µL and 1 mL are being processed. However, no reliable method is available to extract and concentrate the DNA of pathogens, present with 1 cfu or less per mL, from a volume, necessary to allow reproducible PCR results. If one assumes that for a reliably positive PCR at least 5 copies of the target DNA are required, 5 mL of body fluids must be extracted and concentrated in a final volume of 10 µL or less (for a single PCR to be done from the extract). Extraction with a high concentration factor

(i.e. large starting amount and low extraction volume) can be done, but the lower the extraction volume the lower the extraction efficacy. Furthermore, such procedures cannot be adapted for automated extraction. However, in the routine laboratory, which processes a large number of nucleic acid extractions per working day, procedures are usually adapted to automation. If the starting material is blood, the final extract will have as much of human DNA that amplification of the target DNA, even if present in sufficient amounts, is likely being inhibited by the presence of the vast amount of human DNA.

Assuming a detection limit of 5 genomes per PCR (single copy target) and a cfu load of 25 genomes per mL, a standard extraction using column based technology would start with 200 µL of starting material (5 genomes) eluted in 100 µL (giving a final concentration of 1 genome per 20 µL). Standard applications in an amplification volume of 50 µL usually run with 5 µL of extract and must therefore lead to a negative result. If one would instead use a starting volume of 5 mL (125 genomes), and extract in 25 µL the final concentration would be 5 genomes per µL and a standard PCR would result in amplification, even of a single copy gene. This theoretical calculation assumes an extraction efficacy of 100%, which is not realistic. In case, a high cfu load is present in an infected tissue or an organ, standardized protocols, even with starting volumes of 200 µL are sufficient. The available literature about PCR assays for the detection of *Borrelia* from cerebrospinal fluids, synovial fluids or urines highlights the necessity of using large volumes for extraction in order to increase the sensitivity of PCR.

LOW AMOUNTS OF DNA IN LIQUID SAMPLES AND NECESSITY TO USE LARGER VOLUMES FOR EXTRACTION

SCHMIDT *et al.* (1996) [41] used large amounts of urine volume for the detection of *Borrelia* DNA (targeting the flagellin gene in a heminested approach). Starting with 8 mL of urine, concentration of a factor 13 was achieved by dissolving the pellet after centrifugation in 600 µL PBS. A further up concentration of a factor 6 was than achieved by a second centrifugation step after which the pellet was dissolved in a volume of 50 µL. This was mixed with an equal volume of Chelex-100 and 10 µL of the resulting supernatant were used for PCR. The limit of detection when using a nested PCR approach (25 cycles first PCR, followed by 35 cycles of the second PCR) was less than 5 genomes per PCR, equaling 50 borrelia in the original 8 mL urine volume. The extraction protocol was modified later on [38, 45] (DNAzol, starting with 10 mL of urine, the pellet being resuspended in 1 mL, than recentrifuged, and resuspended in 100 µL). Using the same primers as in [41] the limit of detection was also found to be 5 genomes per PCR. Positive results were only obtained after DNA preparation with DNAzol (10 mL starting volume) but not with other methods tested (such as QIAGEN columns, which use 0.2 mL as starting volume). In a subsequent work, the group further analyzed the problem of low DNA amounts in liquid samples by again using urine and demonstrated that a one-step real-time PCR assay for the detection of the *Borrelia* flagellin gene was less sensitive when compared to a nested PCR protocol [46]. While the nested protocol yielded positive PCR results with as low as five *Borrelia* genomes per PCR, the one-step real-time protocol was found to be positive reproducibly, when 50 to 100 genomes (DNAzol extraction) or 500 genomes (QIAamp and Roche extraction kits) were present per PCR (results from spiked urines).

Large volumes of urine (10 - 50 mL) and synovial fluids (1 - 10 mL) were used in another study [28]. Following centrifugation the resulting pellet was washed and subsequently used for an alkaline lysis method. Applying this large volume DNA extraction method to samples obtained from patients with Lyme arthritis and Lyme neuroborreliosis, diagnostic sensitivities of 91% and 87% could be achieved. These high rates of sensitivity can be explained by the use of two types of specimen per patient (synovial fluid/urine or CSF/urine) and the use of two different PCR assays. Consequently, when using low volumes of sample material, the diagnostic sensitivity of PCR was found to be low. ZBINDEN and colleagues (1994) [47] reported two out of twelve patients being positive by PCR when using 50 µL of CSF for DNA extraction.

CERAR *et al.* (2008) [48] amplified DNA from 11.9% of 135 blood- and of 15.4% of 156 CSF-samples obtained from patients with Lyme neuroborreliosis, suscpected LNB and other clinical diagnosis. Two nested PCR assays were used, one, targeting the *rrf-rrl* intergenic region (also known as 5S/23S intergenic region) [10, 11], the other one targeting the *ospA* gene of predominantly American *Borrelia* isolates [7]. Ten of 48 (21%) CSF-samples of patients with neuroborreliosis yielded a positive PCR result in at least one of both PCR assays. For the *rrf-rrl* region, the authors cited a previous work [11]. However, since in [11] only a normal, i.e. not a nested PCR was published, the nature of the outer primer pair (SPA1/SPA2) in the later work [48] remains obscure. The authors concluded, that the detection of *Borrelia* DNA (or RNA) from clinical specimens is far from being standardized. Since real-time assays become more and more a standard procedure, the MIQE guidelines [49] provide a good basis for the future development and publication of these assays.

STANDARDIZATION IS OBLIGATE

The examples discussed above illustrate that the absolute necessity for having a reliable method for nucleic acid amplification techniques (which includes the type or suitable starting material, processing of large volumes if necessary, extraction process, sufficiently validated and accepted primer/probe systems, and finally the amplification and detection), in order to provide unambiguous diagnostic results. That this is not wishful thinking documents the PCR for detecting HIV RNA in patients' blood plasma [16]. Following optimization, two assays define nowadays the standard, reaching a limit of detection of below 40 genomes per mL. The development of such assays needs the engagement of partners from industry. Validation of such (commercialized) assays need clear disease and case definition criteria, meaning, that such assays are perhaps limited to a subset of patients. However, without standardized and rigorously validated PCR assays, the discussion on the necessity of compulsory reporting is obsolete, since reliable reporting of disease cases not only needs an accepted clinical case defini-

tion but also an accepted and validated process of direct pathogen detection (either culture or nucleic acid amplification).

Why further is standardization of PCR as a direct detection method for *Borrelia* DNA important?

RE-IMBURSEMENT SYSTEM (GERMANY)

The following is specific for German patients, only. Detection of *Borrelia* DNA is not subject of reimbursement by the public healthcare insurance provider (collectively named 'statutory health insurance fund', abbreviated SHIF for the remainder of the paper) in Germany. If a patient suffers symptoms of disease, specific for borrelosis, a basic serology (i.e. ELSIA) is reimbursed. If the ELSIA is positive, a conformational test (i.e. Western- or immunoblot) is also reimbursed. Also part of the reimbursement system is the culture of the spirochetes. The overall amount of money granted by the reimbursement system does not cover, however, even the cost of the culture material. If a *Borrelia*-PCR is requested or necessary, however, this has to be paid for by the patient itself. SHIF in Germany takes care for about 70 million people.

For parameters, not yet part of the reimbursement system of the SHIF, a complex routine exists to enable medical progress to be made accessible for those, not being privately insured. If, however, a specific laboratory parameter (usually termed 'patient relevant innovation' or 'medical innovation') is found to be useful by companies, developing novel diagnostics or by medical or scientific associations, these stakeholders are allowed to propose this 'innovation' (by submitting a detailed application) to the so-called "National Association of Statutory Health Insurance Physicians" (German: Kassenärztliche Bundesvereinigung" – KBV[1]), which, in turn performs a rigorous check of the application [50]. If the proposed innovative parameter is found to be important and the application fulfills all criteria, the KBV may than apply for a consultancy claim at the G-BA (The German Health Care System and the Federal Joint Committee, German: Der Gemeinsame Bundesausschuss). The G-BA will than follow a complex routine process to decide whether an innovative parameter can be accepted as benefit for the catalogue of the SHIF. The KBV, which receives the applications for novel or innovative parameters, has published a guideline about the minimum information needed, to submit the application and provides some examples of the necessary information (SCHIFFNER R. Criteria used by the KBV-innovation service for decision on proposals of medical, non-pharmaceutical innovations to the German Federal Joint Committee (G-BA). 2008; Poster T-117 http://www.kbv.de/veranstaltungen/innovationsservice.html (download page for accessing the poster; last accessed: July 16th 2011)).

For the current paper the above outlined procedure to include improvements in medical diagnostics in the catalog of benefits illustrates that only highly standardized and rigorously validated PCR procedures may have a chance of success. Therefore, every effort must be undertaken, to improve PCR performance with the goal to have accepted methods available which allow for reproducible results. The results generated with such standardized and accepted methods need to match the clinical diagnosis. With respect to this, clear criteria are also required to classify whether a given clinical specimen such a CSF is suitable for PCR diagnosis (i.e. sufficient volume of body fluid or size of biopsy, adherence to pre-analytical procedures including time of and conditions during transportation). These criteria exist in principle and clinicians as well as laboratories are advised to use them appropriately.

COMPULSORY REPORTING OF CASES

In the European Community, only a few countries have implemented compulsory reporting for Lyme-disease cases [19]. In Germany, borreliosis is a notifiable disease in five of 16 states; another two states are prepared to start notification. Some organizations in Germany favor a general reporting system. However, clear criteria are required in order to implement an effective reporting. There should be an effective measurement to discriminate between cases which might be a borreliosis (but, in fact, are a different disease, presenting with similar symptoms – and requiring different treatment) and those which are true cases. For the occurrence of an erythema migrans following tick bite being an accepted case definition criterion, reporting and coverage of Lyme disease epidemiology could by enhanced by an optional (!) *B. burgdorferi* PCR from the erythematous lesion. PCR confirmation would lead to a much higher quality of the reporting data and would provide important improvements in case management, since PCR enables the fast and reliable identification of the genospecies involved. An ultimate necessity of such a PCR supported reporting system would be, however, a standardized and validated amplification assay.

CONCLUSION

Without doubt NAAT for the detection of *Borrelia* genetic material in clinical specimens is a highly important diagnostic tool to aid the clinician or general practitioner/office physician in finding or ensuring a definite diagnosis of LB/LD in the suffering patient. Due to the lack of commercially available and sufficiently validated assays, many different PCR protocols are in use. While each published protocol may have its benefit for a specific patient population, a rigorously validated and standardized PCR assay is needed in order to face the actual challenges in diagnosing vector borne infectious diseases. Currently, caution is required when choosing a PCR protocol from the published ones, since – as shown for the *ospA* targeting PCRs – some primers may only detect a subset of the known *B. burgdorferi* strains. An *ospA* PCR with primers designed on older *B. burgdorferi* s.s. *ospA* sequences may be sufficiently specific when used in the US but not in Europe.

While standardization is required regarding the optimal volume of a liquid or solid clinical specimen (i.e. what is the minimal/optimal volume of CSF, urine, tissue to be processed) the Standardization in nucleic acid extraction (i.e. optimization to automated solutions) has not necessarily contributed to better diagnostics. Since automated formats often use comparatively small volumes of specimen to start with (typically 200 µL), the low cfu load of *Borrelia* in clini-

[1] for an English summary about this association please go http://www.kbv.de/78.html

cal specimens might make larger volumes necessary (minimum 1 mL, up to 10 or more mL for urine).

A couple of different micro-organisms are harbored by ticks and many of them may be principally transmitted during tick bite. Although *B. burgdorferi* is the best studied among these organisms, some other may also cause disease, probably with symptoms similar to those seen in LB/LD patients. However; in order to provide the most appropriate treatment for the patient, an accurate diagnosis is needed. While so-called co-infecting or co-transmitted micro-organisms become increasingly appreciated, robust laboratory procedures are required to allow the reliable (and reproducible) detection of the DNA (or RNA) of the infecting micro-organism.

Of particular interest is the novel concept of combining classical PCR on multiple loci with electrospray ionization mass spectrometry (PCR/ESI-MS) [51, 52]. This PCR is currently being commercialized, although as a vector-borne assay, only, but may provide a sensitive and specific PCR method for use in human diagnostics in the near future. Subsequent work by the same group aims on detecting and identifying *Borrelia* directly from blood of patients with erythema migrans (ESHOO M, CROWDER C., ROUNDS M, MATHEWS H, SOLOSKI M, SCHWARZWALDER A, SCHUTZER S, AUCOTT J (2011): Conference abstract O358: "Direct detection of early Lyme borreliosis from whole blood". 21st European Congress of Clinical Microbiology and Infectious Diseases – 27th International Congress of Chemotherapy, May 7th – May 10th 2011, Milan, Italy).

Novel PCR assays, commercial or not, should have been assessed for their clinical and analytical sensitivity and specificity before being used in routine diagnostics. With advanced molecular assays at hand and in companion to clinical diagnostics, robust case definition criteria of acute disease should become accepted, which in turn should allow high quality compulsory reporting.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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