Justyna Dunaj, Anna Moniuszko, Joanna Zajkowska, Sławomir Pancewicz*

THE ROLE OF PCR IN DIAGNOSTICS OF LYME BORRELIOSIS

Department of Neuroinfections and Infectious Diseases
Medical University of Bialystok

ABSTRACT

Molecular biology techniques play a significant role in diagnostics of many infectious diseases. Polymerase chain reaction (PCR) is used to confirm tick-borne diseases e.g. *Borrelia burgdorferi* infection. Extension of PCR method in laboratory diagnostics of Lyme disease (LD) gives a possibility of confirmation of spirochete infection before patient body managed to produce antibodies. Diversity of material, which may be tested (blood, cerebrospinal fluid, skin biopsies, synovial fluid) and possibility of using several conservative genes for *Borrelia* genospecies additionally widen a diagnostic utility of PCR tests. Though high divergence of PCR results obtained in various laboratories is still a significant issue. Hence, standardization of molecular diagnostic in LD is so important.

Key words: *PCR, Borrelia burgdorferi sensu lato, Lyme disease*

INTRODUCTION

Lyme borreliosis is a bacterial infectious disease, transmitted to humans by *Ixodes* ticks. The *Borrelia burgdorferi sensu lato* (*B. burgdorferi sl*) complex consists many of Lyme disease (LD) etiologic factors such as: *Borrelia burgdorferi sensu stricto* (*B. burgdorferi ss*), *Borrelia afzelii, Borrelia garinii* and according to some reports also *Borrelia valaisiana*, *Borrelia lusitaniae*, *Borrelia spielmanii*, *Borrelia bissetti* (1-4).

LD is a multi-system disease, which affects usually skin (*Erythema migrans* - EM), *Acroderamtitis Chronica Atrophicans* - ACA), joints (*Lyme arthritis* – LA), nervous system (neuroborreliosis – NB), but also heart (*Lyme carditis*) and even eyes (1, 3-6).

Annual incidence on LD in Poland increased systematically until 2009, when 10313 cases were registered. 9159 new cases were reported in 2011, (www.pzh.gov.pl). It points to permanent and high risk of *B. burgdorferi* infection in Poland. In the diagnosis of Lyme disease play a significant role: epidemiological interview, an endemic area, where there has been bitten by a tick, the fact of tick bite as well as clinical symptoms occuring in the patient, and laboratory confirmation. Laboratory diagnostic of LD is based on 2-level scheme of immunoserological testing: determination of specific antibodies index by ELISA and confirmation by Western blot or Immunoblot (1, 3, 7-10). Immunoserogical tests should be performed at least 4-6 weeks after tick bite (7, 10) (IgM antibodies appear in 3-4 week, peak after 6-8 weeks; IgG antibodies appear in 4-6 week, peak after 4-6 months). It is therefore necessary to search for diagnostic methods, which give the possibility of earlier confirmation of infection.

THE PCR METHOD IN DIAGNOSTICS OF LYME BORRELIOSIS

Practical application of PCR is the relevant most appropriate in early phase of *B. burgdorferi* infection, when bacteria are transmitted into after tick bite), up to the time, when specific antibodies against bacterial antigens appear (up to 4-8 weeks) (10-14). In early Lyme disease (localized and disseminated) PCR may serve to confirm the diagnosis and detection of co-infections with other pathogens transmitted by ticks (3, 11). In late stage of the disease it might enhance laboratory diagnostics, especially when immunoserological methods are not sufficient (immunoserological defects, accomapanied diseases, intracellular occurence of bacteria, presence of immunological complexes in

* Justyna Dunaj is a scholar of project: „Study, research, commercialize – UMB doctoral candidate support programme ", section 8.2.1 of Operational Programme Human Capital, co-financed by European Union from European Social Fund.

blood) (8). *Ivacic* et al. is also considering the use of PCR in patients with re-infection of B. *burgdorferi sl*, in which traditional immunoserological diagnosis is difficult because of the already existing antibodies (13).

To perform the proper PCR reaction choice of material, way of specimen collection, method for DNA extraction, multiplied targeting sequence, primers sequences, type of PCR method, way of amplicons detection and type of hardware, especially the thermocycler are important (3, 13, 14).

THE MATERIAL FOR PCR EXAMINATIONS

Special attention should be paid to the appropriate way of probe collection for PCR. Short storage time of material and optimal temperature of +4ºC are crucial (3). Use of fixing agents, such as: ethanol, paraffin, formalin (3, 8) and repeated freezing and defreezing of the material or extracted DNA, may lead to fragmentation or even complete degradation of DNA and should be avoided (15).

Skin biopsy. Skin biopsy (3, 15) may be collected from EM lesions (early localized and disseminated changes) just 3 days after tick bite. It is also recommended and for people with ACA (16) (late, chronic changes). *Weber* et al point out the patients with so-called mini EM, having a diameter in the range from 2 cm to 5 cm, which are symptoms of early Lyme disease and may not be subject to further enlargement (17). *Brettschneider* et al. proved that detectability of *B. burgdorferi sl* by PCR is higher in freshly collected or fresh-frozen EM lesions (79%) in comparison with paraffin-embedded or formalin-fixed tissues (44%) (3). Amplification is characterized by high specificity (about 100%), while the sensitivity in EM varies from 36 to 88% (average 69%), in ACA from 54 to 100% - (average 76%) (3).

Joint fluid. Positive PCR in joint fluid of patients with LA may confirm diagnosis, attest to linger infection and occurs in cases of ineffective treatment, it may confirm LA resistant to therapy (3, 9, 11). Specifity of the test is about 100%, while sensitivity 42-100 % (average 78%) (3).

Cerebrospinal fluid (CSF). The lumbar puncture is essential in suspicion of neuroborreliosis. After collection, aseptic CSF has to be investigated for intrathecal antibodies production. It may be also tested with PCR for *B. burgdorferi sl* DNA detection. Because of low number of spirochetes in CSF (8, 18), bacterial affinity to myelin structures (8) and possibility of degradation of genetic material, the negative PCR does not exclude the infection (4, 6, 10, 13). The highest probability of detection of spirochetes DNA in CSF occurs in early neuroborreliosis, (3, 4). It significantly increases if specimen is centrifuged (4, 12). *Lebech* et al. detected *B. burgdorferi sl* DNA in 7 of 14 (50%) patients with early neuroborreliosis and in 2 of 16 (13%) patients with disease lasting for more than 2 weeks (15). According to the guidelines of European Federation of Neurological Societies (EFNS), presented by *Mygland* et al. (19), detection of *Borrelia* spirochoetes in CSF is of value only in diagnosis of very early neuroborreliosis in first 6 weeks, when specific antibodies are absent in patients serum. *Ornstein* et al. (20) noticed a significant role of pleocytosis on PCR results in CSF. In their research *B. burgdorferi sl* DNA was detected in 7 out of 36 (19.4%) CSF with high pleocytosis, while negative results were obtained in all 29 CSF with low pleocytosis (20). *Cerar* et al. (18) detected *B. burgdorferi* sl DNA using primers targeting *OspA* genes and *rrf-rrl* gene intergenic space, in blood of 16 of 135 (11.9%) patients and in 24 out of 156 (15.4%) CSF of patients. Simultaneous presence of spirochoetal DNA in patients' blood and CSF was observed in 3 cases. *Gooskens* et al. estimated the sensitivity of PCR method in diagnostics of neuroborreliosis as 50% and considered PCR as the confirmatory method (4). The specificity of PCR in CSF is high (about 100%), with very divergent sensitivity in various diagnostic centers within the limits between 12-100% (average 38%) (3).

Blood. Easy access to blood collection from every patient after tick bite, independently on presenting manifestations, is the reason why blood seems to be the material of choice for *B. burgdorferi sl* DNA detection. However, low and transient spirochetemia and high spirochoetes tropism to tissues (joints, heart, meninges) may give negative PCR results (1, 3, 6, 10, 12, 18). Also potential inhibitors (heparin, haemoglobin, ethanol, hosts DNA) (18) and method of blood collection influence PCR results. PCR tests in blood are relevant in early disseminated LD, when spirochoetes spread from skin to different organs and tissues (2, 3). *Maraspin* et al. (13) emphasized that spirochetemia is more commonly present after tick bite, than in later stage of disease. *Dolan* et al. (2004) in his study on mice infected with *B. burgorferi sl*, confirmed that spirochoetes detectability is the highest in the early stage of infection (13). In late LD *B. burgorferi sl* DNA detection by PCR in blood has of limited use and always should be interpreted in correlation with the presence of specific anti-*Borrelia* antibodies in patients' serum. Positive PCR results in patients without specific anti-*Borrelia* antibodies are often false positive. *Chmielewska-Badora* et al. in group of 180 patients with diagnosed LD, confirmed *Borrelia* infection by PCR in 20 people (11.1%) and significant positive correlation of PCR result with presence of VlsE antigen in IgG class (21). *Cerar* et al. in group of 48 patients diagnosed as neuroborreliosis detected *Borrelia* DNA targeting *OspA* gene in 10 (20.8%) blood samples and targeting *rrf-rrl* gene intergenic space in 5

(10.4%) cases. In a group of 45 people with suspicion of neuroborreliosis, the presence of *OspA* gene was confirmed in 5 (11.1%) cases and *rrf-rrl* gene intergenic space in 7 (15.6%) patients (18). According to different centers the specifity of PCR is 100%, while sensitivity is 0-100% (average 14%) (3).

Urine. The urine is the easiest material to be taken from patient, but available research indicated low usefulness of this material in LD diagnostic. Presence of various PCR inhibitors and questionable correlations with symptoms decrease the sensitivity of reaction (3, 9, 14, 15). Many examples of nonspecific amplification of PCR products have been described (15). *Lebech* et al. (15) estimated the sensitivity of PCR in urine samples of patients with EM at13 % and in group of patients with neuroborreliosis only in 7%. *Kondrusik* et al. demonstrated lack of *B. burgdorferi sl* DNA in urine samples of all 86 examinated patients with EM (14). Nowadays, all published standards excluded using urine for PCR method, because of low amplification specificity (1, 3, 6, 9, 14).

POLYMERASE CHAIN REACTION

In *B. burgdorferi sl* DNA detection the process of DNA extraction, selection of primers` sequences and conditions of reaction do not differ from standards, whereas selection of targeting sequence and type of PCR may have a significant importance.

Targeting sequence. In *B. burgdorferi sl* targeting gene selection, lack of homology with the DNA of other microorganisms (*Treponema*, *Leptospira*, *Escherichia coli*) and first-of all with human genetic material are the crucial matter (3, 6, 14, 17). There are several, recommended, specific for *B. burgdorferi sl* genome fragments (1, 9), transferred by chromosomes genes: *fla* (22), recA, 16S rDNA (14, 16, 22, 23), p66 (3), *hbb* (4), rpoB, intergenic spacer 5S 23S (19) and plasmid-carried genes: OspA (4, 13, 16, 18), OspB, OspC (16, 24), VlsE (25). .

Types of PCR. Qualitative End-Point type reactions, such as: qPCR (qualitative PCR), nPCR (nested PCR) FEP PCR (Fluorescent End-Point PCR) (3, 4, 11, 14, 25), which detect the presence of *B. burgdorferi sl* DNA are sufficient for LD diagnosis. Quantitative reaction, such as Real-Time PCR, which define the number of *B. burgdorferi sl* DNA copies in collected material, have the same usefulness, but are more expensive (3, 4, 11, 13).

Standard End-Point qPCR in 30-40 cycles multiplies initial matrix DNA one million times, which gives 0.2-2µg of specific genome fragment (3, 6). Nested End-Point PCR (nPCR) is composed of two consequentive PCR reactions. In the second reaction primers' sequences are localized closer to the middle of the amplified fragment. It is characterized by higher specifity, reducing number of false positive results. If both reactions are performed in one tube (locked system), the sensitivity also increases (16, 23). Lee et al. (23) proved that using nested PCR in human specimens increases markedly from 100 to 1000 times the sensitivity of reaction in comparison with standard PCR and allows to detect even single copy of *B. burgdorferi sl* DNA. About 100 spirochoetes are required to detect the infection by conventional PCR. Introduction of internal control in particular specimen probe eliminates false negative results and excludes presence of reaction inhibitors or probability of improper DNA extraction (4, 13).

DIAGNOSTIC DIFFICULTIES

All the procedures of molecular biology techniques must be conducted according to the standards and by adequately selected and properly trained personnel (1, 16, 23). In the interpretation of the test many aspects must be taken into consideration: patient clinical state, diversity and overlap of symptoms, coinfections with other pathogens transmitted by ticks (11). In doubtful cases diagnostic process should be repeated.

Contamination of specimen. At each stage of PCR diagnostics, may happen contamination of specimen: from moment of material collection to detection of amplification product. The potential sources of contamination are: place of material collection or laboratory space, staff, patient and even water used into reagents. The material may be contaminated by e.g. DNA of other pathogens (patients natural bacterial flora), human DNA (when lack of specific primers sequences), other DNA extracts or amplicons from previous PCR reactions, present in laboratory space (3, 6, 11, 14, 24). Contaminations and low specifity of used method lead to false positive PCR results (23). Lee et al. emphasized that the risk of false positive PCR results in *B. burgdorferi sl* DNA detection may lead to wrong diagnosis and cause unsuitable treatment (23). Simultaneously, they denied that nested PCR method is a source of cross-contamination and emphasized that basic causes of contamination are incompetent or negligent personnel. Nested PCR limits the possibility of potential laboratory mistakes and excludes false positive and false negative PCR results (23).

PCR inhibitors. Contamination or inhibition may appear with the same frequency in PCR. PCR inhibitors are: compounds, substances and factors, which are present in specimen, DNA extract or reaction mixture (3, 4, 6, 8, 22). Human haemoglobin, heparin or EDTA interact with a termostable polymerase which catalyzes

PCR reaction by reduction of its cofactor (Mg^{++} ions) (6, 8, 25). *Opel* et al. (25) confirmed that melanin from skin, hair and porfirin derivatives (haem) block specific compounds between primers sequences and matrix DNA, what limits the number of amplificated DNA matrix and may lead to false negative results, especially when EM lesions are examined. Sodium chloride, sodium acetate, isopropanol, ethanol, phenol, SDS or fixing agents, such as: paraffin and formalin inhibits PCR, similarly to some physical factors, e.g. UV transillumination (3, 6, 15, 25). Therefore, the proper DNA extraction process is crucial. It might be combined with purification of extracted DNA, which unfortunately is associated with loss of DNA and additional investment of time and cost (3, 25). Use of internal control in PCR, eliminates influence on amplification inhibitors and reduces numbers of false negative results (13).

Features of *Borrelia burgdorferi* spirochoetes. Individual features of *Borrelia burgdorferi sl* may influence PCR results e.g. short presence in blood, CSF and in other host's body fluids, binding to host's cells (presence of glycosaminoglycans receptors) and colonization of specific loci of organism (skin, synovial membrane, endothelim cells, heart, pericardium, brain and cerebral meninges) (2, 8, 10). It hampers confirmation of infection on molecular level (1, 2). Therefore, negative PCR result does not exclude *Borrelia burgdorferi sl* infection (1). Some other features of *Borrelia burgdorferi sl* make PCR a useful diagnostic method. Bacterial OspC protein in combination with Salp15 protein from tick saliva are facilitating factors for infection (inhibition of dendritic cells function, production of proinflammatory cytokines and activation of B lymphocytes). It extends the time needed for antibodies production, while genetic material of bacteria might be detected by PCR (2).

Antibiotic treatment. One of the most common prelaboratory mistakes is specimen collection after initiation of antibiotic treatment. *Picha* et al. showed that *Borrelia* spirochoetes DNA detection decreases from 58.1 % to 41.7% after the beginning of antibiotic treatment (16). *Kondrusik* et al. (14) indicated that few days of antibiotic treatment does not decrease PCR effectiveness, whereas 4-weeks of antibiotic treatment influences on decreasing detectability. In group of patients without any treatment detectability of *Borrelia* spirochoetes DNA was 73.3% and after 4-weeks course of antibiotics was 52.3%. Similarly, in group of patients after 4-5 days of treatment, detectability of *Borrelia* DNA was 85.7% and decreased to 57.1% after treatment. It should be noticed that positive PCR result confirms a presence of *B. burgdorferi sl* DNA derived from alive or dead bacteria (1, 11). Fragments of bacterial DNA may be detected in 4-6 weeks after antibiotic treatment, therefore PCR control examination is recommended. (14, 22). *Honegr* et al. detected *Borrelia* spirochoetes genetic material in human organism after 4-68 months after antibiotic treatment (23).

Lack of standarization. Great divergence (11) between *B. burgdorferi sl* DNA detection obtained in various scientific and diagnostic centers is a main reason against addition of PCR to LD routine diagnostics (1, 24). Standardization of molecular biology methods and induction of at least two, parallel, independent amplifications are necessary in LD diagnostics, according to *Picha* et al. (16, 25). However, *Cerar* et al. (18) emphasized fundamental meaning of appropriately matched control group in LD diagnostics, which excludes false positive results and confirms high specifity and sensitivity of using PCR method, and it influences on standardization.

DIAGNOSTIC USEFULNESS OF PCR METHOD IN LYME DISEASE-SUMMARY

B. burgdorferi sl DNA detection by PCR widens and speeds up diagnostic possibilities of LD. After *Ixodes* tick bite it is possible to detect even single copy of *B. burgdorferi sl* DNA (4, 13, 22), but it is not a proof of active infection (6). Also negative PCR result does not definitely exclude infection. In the late stage of infection, when specific antibodies should be present, it is possible to receive positive PCR result, especially in an active phase of infection (LA, ACA, NB) and in seronegative patients (immunological defects: congenital and acquired, coinfections, asymptomatic course or latent stage of the disease – activation of infection may appear after months or even years after contact with the vector) (16). PCR method gives additional possibilities in diagnostic process of patients with long-lasting presence of specific antibodies, especially in IgM class (13).

Control PCR examination conducted during and after antibiotic treatment may be helpful in monitoring the effectiveness of anti-*Borrelia* therapy (16, 22).

Diversity of material, which may be used in PCR examinations spreads LD diagnostic possibilities. According to *Stanek* et al. (1) in early stage (EM) and in late stage of the disease (ACA), where skin lesion is a material for *B. burgdorferi sl* DNA detection, detectability is rather high (more than 50-70%). In joint fluid (LA) it is more than 50%, in CSF is rather low (15-30%), and in blood, because of great divergences detectability balance between 0% to 100%. Using urine for LD diagnostics is not recommended.

Introduction of PCR for LD diagnostics gives possibility of detection of infection in its early stage and in patients in whom immunoserological methods fail because of different reasons. Hence, standardization of PCR method and unification of diagnostic procedure schema is essential.

REFERENCES

1. Stanek G, Fingerle V, Hunfeld KP et al. Lyme borreliosis: Clinical case definitions for diagnosis and management in Europe. Clin Microbiol Infect 2011; 17: 69-79.
2. Mączka I, Tylewska-Wierzbanowska S. Cykl krążenia krętków *Borrelia burgdorferi* w środowisku. Post Mikrobiol 2010; 49(1): 25-32.
3. Aguero-Rosenfeld ME, Wang G, Schwartz I et al. Diagnosis of Lyme Borreliosis. Clin Microbiol Rev 2005;18(3):484-509.
4. Gooskens J, Tmpleton KE, Claas C et al. Evaluation of an internally controlled real-time PCR targeting the OspA gene for detection of *Borrelia burgdorferi* sensu lato DNA in cerebrospinal fluid. Clin Microbiol Infect 2006;12: 894-900.
5. Zajkowska J, Pancewicz SA, Kondrusik M et al. Lyme borreliosis – The tick-borne disease. Adv Pol Acarol 2007; Warszawa, Wyd. SGGW: 458-468.
6. Schmidt BL. PCR in laboratory diagnosis of human. Clin Microbiol Rev 1997; 10(1):185-201.
7. Pancewicz SA, Zajkowska JM, Kondrusik M et al. Znaczenie epidemiologiczne i kliniczne chorób przenoszonych przez kleszcze. Monografia red. dr hab. Jan F. Żmudziński. Warszawa 2010; 107-125.
8. Gąsiorowski J, Witecka-Knysz E, Knysz B et al. Diagnostyka boreliozy. Med Pracy 2007; 58(5):439-447.
9. Reed KD. Laboratory Testing for Lyme Disease: possibilities and practicalities. J Clin Microbiol Rev 2002; 40(2):319-324.
10. Zajkowska J, Pancewicz S, Grygorczuk S et al. Neuroborelioza- wybrane aspekty patogenezy, diagnostyki i leczenia. Pol Merk Lek 2008; 24(143): 453-457.
11. Swanson SJ, Neitzel D, Reed KD et al. Coinfections acquired from *Ixodes* ticks. Clin Microbiol Rev 2006; 19(4): 708-727.
12. Maraspin V, Ogrinc K, Ruzic-Sabljić E et al. Isolation of *Borrelia burgdorferi* sensu lato from blood of adult patients with borrelial lymphocytoma, Lyme neuroborreliosis, Lyme arthritis and acrodermatitis chronica atropicans. Infection 2011;39:35-40.
13. Ivacic L, Reed KD, Mitchell PD et al. A Light Cycler TaqMan assay for detection of *Borrelia burgdorferi* sensu lato in clinical samples. Diagn Micobiol Infect Dis 2007; 57:137-143.
14. Kondrusik M, Grygorczuk S, Skotarczak B et al. Molecular and serological diagnosis of *Borrelia burgdorferi* infection among patients with diagnosed erythema migrans. Ann Agric Environ Med 2007; 14:209-213.
15. Lebech AM. Polymerase chain reaction in diagnosis of *Borrelia burgdorferi* infections nd studies on taxonomic classification. APMIS 2002; 105:1-40.
16. Picha D, Moravcova L, Holeckova D et al. Examination of specific DNA by PCR in patients with different forms of Lyme borreliosis. Internat J Deramtol 2008; 47:1004-1010.
17. Weber K, Wilske B. Mini Erythema Migrans – a sign of early Lyme borreliosis. Dermatology 2006; 212:113-116.
18. Cerar T, Ogrinc K, Cimperman J et al. Validation of cultivation and PCR methods for diagnosis of Lyme neuroborreliosis. J Clin Microbiol 2008; 46(10): 3375-3379.
19. Mygland A, Ljostad U, Fingerle V et al. EFNS guidelines on the diagnosis and management of European Lyme neuroborreliosis. Eur J Neurol 2010; 17: 8-16
20. Ornstein K, Berglund J, Bergstrom S et al. Three major Lyme *Borrelia* genospecies (*Borrelia burgdorferi* sensu stricto, *B. afzelii, B. garinii*) identified by PCR in cerebrospinal fluid from patients with neuroborreliosis in Sweden. Scand J Inf Dis 2002; 34: 341-346.
21. Chmielewska-Badora J, Cisak E, Wójcik-Fatla A et al. Correlation of tests for detection of *Borrelia burgdorferi* sensu lato infection in patients with diagnosed borreliosis. Ann Agric Environ Med 2006; 13: 307-311.
22. Honegr K, Hulińska D, Beran J et al. Long term and repeated electron microscopy and PCR detection of *Borrelia burgdorferi* sensu lato after antibiotic treatment. Centr Eur J Publ Health 2004;12(1): 6-11.
23. Lee SH,Vigliotti VS, Viggliotti JS et al. Increased sensitivity and specificity of *Borrelia burgdorfer*i 16S ribosomal DNA detection. Am J Clin Pathol 2010; 133:569-576.
24. Jones KL, Glickstein LJ, Damle N et al. *Borrelia burgdorferi* genetic markers and disseminated disease in patients with early Lyme disease. J Clin Microbiol 2006; 44(12)4407-4413.
25. Opel KL, Chung D, McCord BR. A study of PCR inhibition mechanisms using real-time PCR. J Forensic Sci 2010;55(1): 25-33.

Received: 13.11.2012
Accepted for publication:31.12.2012

Address for correspondence:
Justyna Dunaj
Department of Neuroinfections and Infectious Diseases
Medical University of Białystok
Żurawia 14 Street, 15-540 Białystok, Poland
Tel.+48 (85) 7409514
e-mail:neuroin@umwb.edu.pl