

PART I. DISEASES AND PROBLEMS DISTINGUISHED BY WHO AND FAO  
DZIAŁ I. CHOROBY I PROBLEMY WYRÓŻNIONE PRZEZ WHO I FAO

CURRENT METHODS USED TO IDENTIFY AND GENOTYPE SPIROCHAETES  
*BORRELIELLA BURGdorFERI*

AKTUALNE METODY IDENTYFIKACJI ORAZ GENOTYPOWANIA KRĘTKÓW  
*BORRELIELLA BURGdorFERI*

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- D. Data interpretation  
interpretacja danych
- E. Preparation of manuscript  
przygotowanie artykułu
- F. Literature analysis/search  
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Summary

Lyme disease, as one of tick-borne diseases, has been a current epidemiological problem in Poland and in the world for several years. The proportion of borreliosis infections caused by *B. burgdorferi* spirochaetes is increasing. Difficulties diagnosing this disease with conventional methods have led to growing molecular biology research aimed at developing improved diagnostic tools. Molecular biology methods include all techniques that allow the detection and analysis of nucleic acids. Among them there are methods based on PCR reaction, molecular typing methods (MLST), new generation sequencing (NGS). The current development of this field gives great research opportunities. With regard to *B. burgdorferi* spirochaete, molecular biology is used to: confirm Lyme borreliosis, identify and type *Borrelia* spirochaetes, detect them in tick vectors or intermediate hosts, as well as to identify co-infections between different *Borrelia* species and other tick-borne pathogens. They are meant to significantly improve diagnostic analyzes. This paper reviews the current methods used for the detection and identification of *B. burgdorferi*. Molecular mechanisms for the survival of spirochaetes in the host, infection vectors and clinical picture of Lyme disease were also discussed.

**Keywords:** DNA, PCR, RNA, *Borrelia burgdorferi*, genospecies

Streszczenie

Borelioza, jako jedna z chorób odkleszczowych, od kilku lat stanowi aktualny problem epidemiologiczny w Polsce i na świecie. Wzrastający odsetek przypadków zakażeń boreliozą, chorobą wywołaną przez krętki *B. burgdorferi*, bardzo trudnych do zdiagnozowania metodami konwencjonalnymi, powoduje stale powiększające się zainteresowanie najnowszymi technikami z dziedziny biologii molekularnej. Metody biologii molekularnej obejmują wszystkie techniki pozwalające na wykrywanie i analizę kwasów nukleinowych. Wśród nich wyróżnia się metody oparte o reakcję PCR, metody typowania molekularnego (MLST), sekwencjonowanie nowej generacji (NGS). Aktualny rozwój tej dziedziny daje ogromne możliwości badawcze. W odniesieniu do krętków *B. burgdorferi* biologię molekularną wykorzystuje się do: potwierdzenia boreliozy z Lyme, identyfikacji i typowania krętków *Borrelia*, wykrycia ich w wektorach kleszczowych, czy gospodarzach pośrednich, a także przy stwierdzeniu koinfekcji między różnymi gatunkami *Borrelia* i innymi patogenami przenoszonymi przez kleszcze. Mają one w znacznym stopniu usprawnić analizy diagnostyczne. W niniejszej pracy przedstawiono przegląd aktualnych metod umożliwiających wykrycie i identyfikację *B. burgdorferi*. Omówiono również molekularne mechanizmy umożliwiające przetrwanie krętków w organizmie gospodarza, wektory zakażenia oraz obraz kliniczny boreliozy.

**Słowa kluczowe:** DNA, PCR, RNA, *Borrelia burgdorferi*, genogatunki

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## Introduction

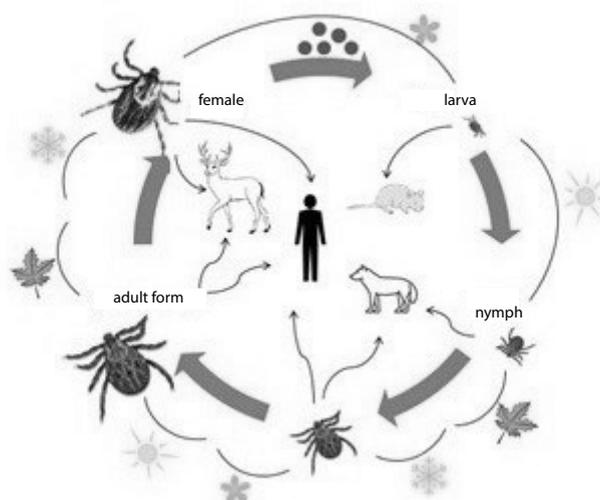
Ticks are currently one of the most widespread ecological vectors causing illnesses to humans and animals. To date, around 900 species of these arachnids have been described worldwide, and in Poland about 20 species have been identified [1]. The reason for the constantly growing number of ticks is global warming, short and mild winters, higher air humidity and long summers. In turn, the reason for the increased occurrence of tick-related diseases is increased outdoor activities, such as walks in meadows and wooded areas associated with a healthy and active lifestyle [2].

The species causing borreliosis, one of the diseases transferred by ticks with known human pathogenicity are *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. bissetii*, *B. spielmanii*, *B. valaisiana*, *B. lusitaniae*, *B. bavariensis*, *B. kurtenbachii*, all part of the wider *Borreliaceae* family. In North America, only one species: *Borrelia*, *B. burgdorferi sensu stricto* occurs with confirmed pathogenicity for humans while in Europe four species have been identified including *B. garinii*, *B. afzelii*, *B. spielmanii* and *B. bavariensis*. Pathogenicity of three other European species: *B. valaisiana*, *B. lusitaniae* as well as recently described species *B. finlandensis* [3] has not been confirmed [4]. Hitherto, the main species representing microorganisms from this group is the *Borrelia burgdorferi* species. Currently, the *Borreliaceae* family is divided into two distinct groups of species which cause different diseases. These groups also have different genetic, genomic and phenotypic compositions. The *Borrelia* species includes clinical and epidemiological factors causing relapsing fever (RF). *Borrelia* is a new species name of the spirochaetes causing Lyme disease (LD) *B. burgdorferi* as well as other human diseases [5].

*B. burgdorferi* spirochaetosis is a Gram-negative bacteria that have a helical, irregular shape and move through cilia actions [6]. The *Borreliaceae* family is diversified and includes many genospecies. They consist of a group of bacteria causing Lyme disease (*B. Burgdorferi*) and other members are responsible for RF (*B. miyamotoi* and *B. lonestari*). Recently, a new spirochaetes group from the *Borreliaceae* family has been identified. It has not been characterised phylogenetically but is associated with the occurrence of Lyme disease in reptiles [7]. The tick itself may be infected with various species of the *Borreliella* and *Borrelia* as well as other pathogens with the simultaneous possibility of transferring them into animals and humans [8]. Epidemiological reports show that in the period from January 1 to December 15, 2018, the occurrence in Poland was 20,150, 52.46 per 100,000 inhabitants. The most recent data show that these values increased to 20,9614 and 53.66 respectively, from January 1 to December 15, 2019. In 2018, the biggest increase occurred in podlaskie, małopolskie, lubelskie and warmińsko-mazurskie voivodship [9, 10].

## Infection vectors

The main spirochaetes infection vector is ticks. The species that occur most commonly in Poland, and other parts of Europe and Asia, are *Ixodes ricinus* – sheep tick and *Dermacentor reticulatus* – marsh tick. These arachnids can transfer various zoonotic pathogens such as bacteria, viruses or protozoa. During feeding the tick may transfer pathogens to its host or become infected [11]. Ticks can get infected relatively easily. The main factors are, firstly that the *B. burgdorferi* reservoir consists of rodents, insectivorous mammals, mainly from Muridae and Arvicolinae families and the carriers of these pathogens and mammals belonging to the Cervidae and Canidae families, birds and lizards [11, 12]. Secondly, the infection of ticks can occur at every stage of their life cycle. Larva, as the initial development stage, can acquire *B. burgdorferi* (and other pathogens) while feeding on small rodents. Infection can last in arachnids for their entire life cycle (around 2 years). Nymphs can transfer bacteria to larger mammals, birds, and people and after transformation into an adult tick, they can still infect their hosts [13] (Figure 1).



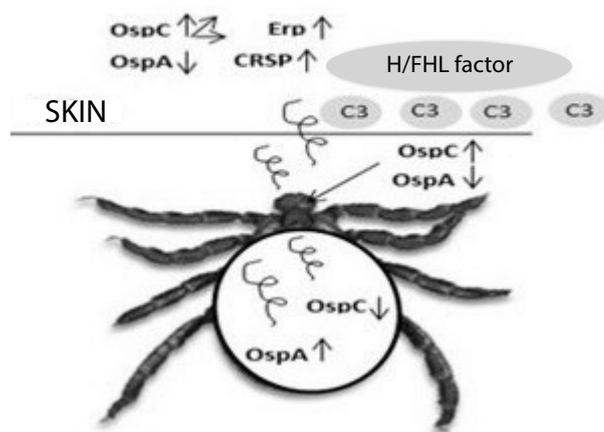
**Figure 1.** The development cycle of a tick and its hosts

Source: Author's compilation according to WSSE in Warsaw [14].

Not every tick is infected and not every bite results in illness. The bacteria can enter the organism of the host directly or indirectly. Direct transmission does not play an important role in transferring spirochaetes *B. burgdorferi* between the tick and humans. The most effective method of pathogen transfer is through saliva during a bite. Pathogen contact occurs in the midgut during digestion and is transmitted to the salivary gland and saliva during a tick bite. Indirect transmission consists, for example in rubbing living bacteria located on the excrements of infected arachnids into the skin [15].

### **Molecular survival strategies of *Borrelia burgdorferi***

*B. burgdorferi* can survive in the host and spread throughout tissues. This is achieved through different adaptation strategies. These aim to ensure colonization and to avoid the host's immune system. These processes occur at the molecular level. *Borrelia* proteins are encoded by plasmid and chromosomal genes and their regulation is modulated by environmental factors of the tick and host. Outer Surface Proteins (Osp) play a significant role in pathogenesis. OspA is an "anchor" for the spirochaete which enables colonization in the tick's intestine. *Borrelia* in an arachnid's intestine decreases the regulation of OspA and increases regulation of OspC – a lipoprotein which correlates with bacterial migration. Because of this, OspA is important for interaction with the host. Expression of outer surface proteins allows spirochaetes to move from the intestine to the salivary glands of ticks and then to the organism of the host [16]. When present in the tick's salivary glands, the biggest obstacle for *Borrelia* bacteria is first line defense of the immune system in the form of the complement cascade (C3) which protects from pathogen transmission. Next, *B. burgdorferi* obtains outer surface proteins (CRASP) and Erp proteins which inhibit the function of the immune system by binding factor H or FHL (factor-H-like). Factor H regulates the function of the complement cascade. Factor FHL is a co-factor of C3 convertase which facilitates fission and binds the proteins from the OspE group. Inhibition of the complement cascade allows spirochaetes to transfer and spread the infection [16, 17] (Figure 2).



**Figure 2.** The regulation of gene expression in *B. burgdorferi*

Source: Author's compilation according to Singh et al. [16].

Another survival mechanism of *B. burgdorferi* involves genetic recombination in the gene locus of the core protein sequence (*vls*). The *vls* expression site is ViSE. The recombination causes alterations in the antigenicity and allows further deception of the host's immune system [16].

In order to avoid immune responses from the infected organism, some strains of spirochaetes show polymorphism of lipoproteins. This means that they can alter their coding genes sequences e.g. OspC. These processes lead to genomic alterations, antigenic diversity, and omnifarious gene expression. All this provides opportunities for *Borrelia* spirochaetes to weaken the immunologic response of the host's organism and maintain chronic infection [16, 18].

## Lyme borreliosis

Tick-borne diseases have been a serious epidemiological problem for several years in Poland and worldwide. Each year an increased number of infections are recorded. Many individual diseases in this group are still diagnosed as Lyme disease, where the aetiology is the above-mentioned spirochaete *Borrelia burgdorferi* [19]. Analysis of the National Institute of Public Health shows the increasing number of infections with Lyme disease as well as the growing number of patients hospitalized because of this illness [10].

Lyme disease is a bacterial infectious disease that is difficult to diagnose due to the differentiation of symptoms and may include skin, musculoskeletal, nervous system or cardiac symptoms [20]. The incubation period is between 2 to 4 weeks and the disease is characterized by three stages:

- Early localized Lyme disease in which erythema migrans (EM) is characteristic. This alteration has a form of a growing spot and appears in 60-70% of infected after 3-30 days in the place of tick's sting. The erythema is accompanied by flu-like symptoms: higher body temperature, headaches, muscle, and joint pains, general frailness and malaise [21].
- Early disseminated Lyme disease is the second phase of the disease and mainly affects the cardiovascular system. Evidence of the presence of spirochaetes in blood, the so-called spirochaetemia is multiple erythema migrans. This stage of the disease is characterized by acute joint pain, myocarditis but also apparent symptoms originating from the nervous system, e.g. facial nerve palsy occurs [22].
- Late Lyme disease is the last stage of the disease where symptoms become chronic. The pathology of the nervous system is defined neuroborreliosis which in the advanced stage causes cranial nerve palsy, meningitis, neuritis, and stroke-like symptoms. Chronic arthritis also occurs. Within the scope of dermatological diseases, acrodermatitis chronica atrophicans (ACA) is observed. Symptoms may also include severe limb pains and inflammation [23].

The clinical symptoms of Lyme disease are varied and can be similar to other diseases. For this reason diagnosing the disease in its early stage is difficult. On the other hand, the constant development in diagnostic methods creates a wider range of possibilities of identification, confirmation, and implementation of effective treatment.

## Lyme disease diagnostic methods

The clinical diagnosis of Lyme disease consists in confirmation or rejection of the occurrence of *B. burgdorferi* spirochaetes or specific antibodies in the examined material. Diagnostic methods can be divided into indirect and direct. Indirect methods include serological tests consisting in the identification of specific antibodies in serum. Direct methods use the identification of infection factors. This group includes methods based on PCR reaction (bacteria DNA amplification) and proliferation of bacteria on microbiological culture media [24].

Biological sample used for testing is typically blood serum but also cerebrospinal fluid, synovial fluid, biopsy material, skin or tissue depending on the chosen diagnostic method.

## Serological methods

Serological diagnostic methods are based in the correct identification of the type of immunological response of the immune system. Initially, each type of infection causes a non-specific and inherent response. The further stages of the immune response of an organism are associated with the infectious factor. Bacterial infection are primarily associated with specific antibody responses, between 2 and 3 week these are early antibodies -IgM. Their peak occurs 2 month after infection. At the next stage, IgM levels decrease and the production of late antibodies IgG begins. Their titer in an organism can be observed in the next years [25]. Laboratory diagnostic of Lyme disease is based on screening tests and analysis of titer-specific antibodies in the patient's serum. The tests are characterized by widespread access, short waiting time, high sensitivity and on the other hand low specificity. Further, it is possible to obtain false-positive results due to cross-reactions [26]. Interpretation of serological results can be difficult due to antigenic complexity of different species of *Borrelia*, differences in immunological potential, and individual immune response of the patient [27].

## The analysis of certain serological tests

Screening test – enzyme-linked immunosorbent assays (ELISA) is the first step in the diagnosis of Lyme disease. This test detects antibodies against spirochaetes *Borrelia* and differentiating the classes of immunoglobulin antibodies. This allows selection of patients who require further diagnosis. However, this test is not used to confirm the disease [28]. Obtaining a negative result does not constitute a basis to fully exclude the infection, especially if the blood was taken too early after the contact with a tick. All positive and inconclusive results require a Western blot which is an infection-confirming test. Western blot's are used to verify the results of the screening test. This method is characterized by higher specificity than ELISA but also lower sensitivity, Western blots are based on the use of the differences in weight and electrophoretic motility of the analyzed proteins. A band from the complete set of *B. burgdorferi* antigens constitutes the control. Characteristic bands are shown on the strip at the moment of binding the antigen of bacteria causing Lyme disease with antibodies. This confirms that the result is positive. One particular band is related to a certain bacterial protein. The following antigens are usually detected: VlsE – cell wall lipoprotein (*B. afzelii*, *B. burgdorferi*, *B. garinii*), p83 – protein connected with the cell wall, flagelin – p41, outer surface protein of the *Borrelia* bacteria p39/BmpA, p31/OspA, p30, p25/OspC, p21, p19, p17/DbpA [29]. The band shown in the flagellin antigen for the IgM antibodies suggests a false-positive result because this protein occurs also in other pathogens e.g. *Helicobacter pylori* [30].

## The limitations of serological methods

One limitation of serological methods is antigenic polymorphism of *B. burgdorferi*. It is important to choose the correct genospecies of the bacteria to prepare the test in order to avoid cross-reactions of antigens or reactivity to the patient's serum. A challenge in the diagnosis of Lyme disease is also the lack of differentiation of the current infection from a previous infection, despite having detected the specific antibodies. During the diagnosis of Lyme disease, none of the above-mentioned stages should be neglected. This means that the tests should not be used individually. Another limitation of serological methods are false-positive or false-negative results. This is due to cross-reactions or early testing. IgM antibodies appear after 2-3 weeks from the moment of infection. Identifying *B. burgdorferi* antibodies is also difficult in the case of coexisting autoimmune diseases. This happens because of the increased titer and lack of association with the stages of the disease. As was mentioned above ELISA and Western blot tests are characterized by low sensitivity to early-stage Lyme disease [31]. The entire clinical picture of the patient and coexisting diseases are significant for interpretation of the result. Developing general standards of laboratory diagnoses of Lyme disease which would allow reproducible

serological methods, reliable results and reducing factors which can interfere with the diagnostic processes are still being conducted.

According to the guidelines of the Polish National Chamber of Laboratory Diagnosticians (Krajowa Izba Diagnostów Laboratoryjnych) the factors influencing the serological tests are also rheumatoid factor occurring in the serum which may cause false-positive results in the IgM class; high concentration of IgG antibodies which inhibit the binding of IgM; iron ions and the occurrence of hemolysis in the sample which makes the interpretation of the result impossible; bacterial contamination; freezing and thawing of the sample [32].

### Microbiological methods

Microbiological diagnosis of Lyme disease consists of *in vitro* breeding and identification of *Borrelia* spirochaetes. The *in vitro* breeding is a routine diagnostic method and its sensitivity depends on the number of bacteria in the tested biological material and their species. Barbour-Stonner-Kelly's medium is used to ensure suitable conditions for *B. burgdorferi*. To date, a relatively low percentage of positive samples were reported of which the majority were skin biopsies. The basic disadvantage of this method is the long proliferation time (up to 3 months). Further, lack of growth does not allow exclusion of the infection. Proliferation of spirochaetes is done in case of obtaining inconclusive serological results and with the presence of Lyme disease symptoms [33, 34].

### Molecular methods

Biomolecular methods include all techniques allowing the detection and analysis of nucleic acids. Current developments in this field provide many research possibilities. In relation to the *B. burgdorferi* spirochaetes molecular biology is used in the following aspects: confirmation of Lyme borreliosis, identification of types of the *Borrelia* spirochaetes as well as their detection on tick vectors or in indirect hosts. Due to modern techniques, it is possible to ascertain the coinfections between different species of *Borrelia* and other pathogens carried by ticks [35].

During the last decade methods based on PCR are used to detect the DNA of *B. burgdorferi*. A significant stage in developing these techniques is the design of oligonucleotide primers which are complementary to the DNA of *B. burgdorferi*. The melting temperature of the primers should be complementary within the pair and the difference of temperatures in reference to the target sequence not higher than 20°C. Oligonucleotides should be constructed of multiple G+C pairs and their length should be 15-30 nucleotides. Attention should also be paid to the fact that primers should have stable 5' endings and they should not be complementary by the ends of the 3' chain.

Tests can be carried out on various biological material including blood, cerebrospinal fluid, skin biopsy, synovial fluid and tick's tissue [36]. The quality of the tested sample is essential for molecular methodology. The period of collecting the sample, storing and transport is also important. Before proceeding with analysis the process of DNA extraction should be optimized, solid tissue should be well-homogenized and treated with K proteinase to ensure quality and efficiency of the isolated genetic material. The key to the success of the molecular methods is the choice of the best target sequence for DNA amplification which would allow the identification of all species from the *Borreliaceae* family [35, 37].

The *borrelia* genome consists of a linear chromosome containing around 910 kpb (thousands of base pairs) on which the majority of genes together with linear and circular plasmids (600 kpb DNA) with lipoprotein coding genes which undergo expression are located. Molecular tests were carried out with the application of various *Borrelia* genes including 16S rRNA, tandemly repeated gene 23S and 5SrRNA, gene *p66*, *ospA*, *ospB*, *ospC* as well as *fla*. Some of them are provided in ready-made, commercial analyses sets [38, 39]. In the diagnosis of *Borrelia* and *Borrelia* spirochaetes, PCR-based molecular methods are often directed at these genes. Among others, Weiner et al. analyzed the occurrence of *B. burgdorferi* in ticks from the region of Tarnopol in Ukraine. Primers for 5S rRNA and 23S rRNA genes which occur tandemly in the genome and are a part of the ribosomal RNA were designed from PCR amplification [40]. Other researchers in their works carried out a chain reaction of the polymerase for 16S rRNA gene which also belongs to the ribosomal RNA but in the *B. miyamotoi* species [41]. PCR molecular analyses of the *Borrelia* spirochaetes are often directed at the flagellin gene. Research conducted in Szczecin, in which fragments of *flaB* gene were amplified out of the *I. ricinus* isolate have demonstrated the occurrence of the following species in their tissue: *B. burgdorferi*, *B. garinii*, *B. afzelii* and *B. miyamotoi* [42]. Genotyping of the *B. burgdorferi* spirochaetes with the application of the *p66* gene and genes of surface proteins from ticks of the *Ixodes persulcatus* and *Ixodes pavlovskyi* species was conducted by Sabitova et al. in Western Siberia and Mongolia [43].

*Borrelia* spirochaetes are highly diversified. Therefore, genotyping aimed at identification of particular species, analyses of plasmid profiles and the entire genome plays a significant role. This type of analysis is vital in epidemiological, clinical and evolutionary research. Nowadays, many methods based on genetic characteristics of the pathogen are available. They are used in the diagnosis of Lyme disease on a large scale. The choice of the appropriate research protocols depends on the physical, chemical and biological properties of the tested sample, features of the isolated microorganism and the assumed goal of the analysis [37, 39].

### PCR reaction-based methods

**Polymerase chain reaction – PCR** consists of DNA amplification of the *Borrelia* spirochaetes. This reaction involves genetic material and requires the known sequences of the target gene. Classic PCR consist of three stages of reaction repeated a certain number of times: denaturation, annealing, and elongation. Each stage has a certain temperature profile, number of repetitions and duration. During the reactions, the amount of DNA is amplified at an exponential rate. This allows obtaining a large number of copies of the analyzed fragment of the gene in a short time [44-46]. Visualization of the results of classic PCR is obtained by electrophoresis in agarose gel. Electrophoresis separates sequences of the nucleic acids, the products of PCR, and proteins across an electric field according to their mass, structure, and load. PCR products appear in the gel as distinct bands. An appropriate fluorescent dye which creates a complex with the DNA, in this case the genetic material of the *Borrelia* spirochaetes, is visible UV light. The bands in the gel are compared with knowns controls used for interpretation of results [46]. The advantages of this molecular method include the relatively short amount of time and a low amount of genetic material required for this test. Classic PCR is characterized by high sensitivity [45].

Classic PCR is a qualitative method. Its refinement has included creating a quantitative technique that allows monitoring the increasing product at each stage of the reaction. *Real-Time* PCR together with the analysis of melting curve of the amplified products is a tool which is used more often in the analysis of Lyme disease in comparison to the classic PCR. Monitoring reaction products is possible due to fluorescent marking of primers, hybridization probes and function of fluorescent dyes. Fluorescent markers cause an alteration in the received signal depending on the amount of product. The stronger it is, the more synthesized amplicon is generated. The basic dye used in the fluorescence detection is SYBR Green I which emits light upon binding with the double strand of DNA. Unfortunately, despite the cost-effectiveness of this method, the basic disadvantage is the fact that the dye intercalates with non-specific PCR products which take on the double-strand form [47]. An important factor in PCR reactions in which hybridization probes are used is the adjustment of the conditions of melting temperatures ( $T_m$ ) of primers and probes. The melting temperature of probes should be higher ( $5^\circ\text{C}$ - $10^\circ\text{C}$ ) than the melting temperature of primers. Oligonucleotides marked with fluorochrome on 5' and 3' ends constitute molecular probes used for fluorescence detection, The commonly used 5' dyes are: FAM (6-carboxyfluorescein), TET (tetrachloro-6-carboxyfluorescein), VIC (registered name) [46, 48]. The function of molecular probes is based on the phenomenon of energy transfer from fluorochrome which absorbs energy to fluorochrome which emits light. Muting this emission is possible due to the function of the extinguisher. Dyes which are commonly used are TAMRA (carboxylotetramethylrodamine) and DABCYL (4-(4'-dimethylamino-fenylazo) benzoic acid). Many types of molecular probes which may be used in PCR reactions are currently available on the market. The most popular are double marked single probes called TaqMan [46]. The melting curve of the amplified products allows valuable information to be obtained about the reaction. It appears together with the increase of temperature and fluorescence graph and after obtaining the temperature allowing detachment of DNA strands ( $T_m$ ), the fluorescence decreases [48, 49].

*Real-time* PCR with the melting curve analysis is a tool facilitating the identification of *Borrelia* spirochaetes. *Borrelia* genes including *p66*, *recA* and *hbb* gene which allows the distinction of the largest number of species, protein-coding *Hbb* gene which belongs to the family of proteins similar to histones are used for this process [38, 50]. Research by Ćakić et al. showed real-time PCR was conducted based on the *hbb* gene. Out of 36 isolated strains, 44.4% was identified as *B. lusitaniae*, 36.1% as *B. afzelii*, 13.9% as *B. garinii* and 5.6% as *B. valaisiana* [51]. An additional advantage of real-time PCR with melting curve analysis in comparison with the classic PCR is the possibility to observe increase product formation at each stage.

Many commercial sets allowing the detection of *Borrelia* spirochaetes and other tick-related pathogens are available on the market. Work this range of products requires appropriate test equipment and isolated DNA with the omission of the stage of primer projection and fluorescence marking which improves the process. The results show which sample contains the target DNA of the *Borrelia* spirochaetes. Lejal et al. used this methodology for the analysis aimed at detecting pathogens in the midgut and salivary gland of *Ixodes ricinus* ticks, determination by their migration and coinfection [52].

*Borrelia* spirochaetes typing by means of the amplification using PCR in the case of primers of similar sequences may be distorted due to the occurrence of cross-reactions between species. An alternative in this case is another technique based on PCR which allows the analysis of **polymorphism of the length of restriction fragments, known as PCR-RFLP**. In the first stage, amplification of the target DNA fragment occurs. Gene *16S rRNA* and intergenic space *22S-5S rRNA* is usually analyzed in *B. burgdorferi* due to its unique structure. The next step is to cut the amplification product with the restriction enzyme *Mse I* which results in DNA fragments characteristic for single *B. burgdorferi* species. The PCR-RFLP method was used to identify the *B. burgdorferi* and *B. miyamotoi* species in *Ixodes ricinus* ticks in research by Kubiak and colleagues. In their study the positive products of PCR were subject to etching with another restriction enzyme – *HpyF3I* and then separating in the agarose gel [53]. Generally, PCR-RFLP has many advantages. First and foremost it is unambiguous, has a wide range of species identification, and direct biological samples can be used for testing – tissue or bodily fluids [46].

Commercial PCR sets used for detection and differentiation of DNA of the *Borrelia* species are often based on **flagellin gene analysis**. It is chromosomal and certain fragments are highly conservative and in other parts, differentiated which permits identification of *Borrelia* species [36, 38].

**Analysis of external surface protein OspC** is also used in molecular tests of *B. burgdorferi* strands. As mentioned previously, the *OspC* gene is located on a plasmid and the protein itself participates in the humoral response of the immune system during infection by the pathogen. The region of about 600bp is used in genotyping and then it undergoes amplification and sequencing. This technique allows the identification among strains of 21 different types of *B. burgdorferi sensu lato*. The practice of this method is complicated due to high amino acid polymorphism of the *OspC* protein and requires trained personnel. On the other hand, due to this high polymorphism, this technique is often used in the population study within the species [38, 54].

PCR based methods used to identify species from the *Borreliaceae* family include RAPD-PCR – an analysis of randomly amplifies polymorphous DNA. A single primer (with random sequence), containing 50-80% of G-C is used for the amplification of genetic material. The primer during the PCR reaction attaches to the DNA in the place of homologous, randomly located sequences. This technique is characterized by a lower number of reaction cycles and lower temperatures of attaching of primers which stabilized the elongation of short fragments. In the case of RAPD-PCR products which visualization is carried out by the electrophoretic separation are developed. Bands in gel determining the products of the same molecular weight characterize single, certain species whereby they are absent in other strains. The success of the method depends on the length of the primer and the size of the studied genome. There are differences in complement sequences depending on the primer used in the reaction between the species of the *Borreliaceae* family. The reason for this is point mutations within sequences. RAPD-PCR also allows analysis of the genetic diversity of tick species. Recently, this method was used by Al-Deeb and Enan who studied *Hyalomma dromedarii*, which does not appear in Europe, utilizing 13 decamer primers [55]. The RAPD-PCR technique is closely correlated with the operation of molecular markers MAAP (Multiple Arbitrary Amplicon Profiling) termed a **genomic fingerprint**. Due to this, random amplification of DNA fragments occurs. In the differentiation of the *Borrelia* species, the **plasmid fingerprint** can also be distinguished. This method allows identification of spirochaetes on the basis of the diverse number and size of plasmids [46].

### Multi-locus sequence typing (MLST)

MLST is a technique that requires the amplification and sequencing of housekeeping genes that code for core metabolic enzymes. For *B. burgdorferi* the following 8 genes are used in typing: *clpA* (a subunit of protease A), *clpX* (a subunit of protease X), *pyrG* (synthesis of cytidine triphosphate – CTP), *nifS* (aminotransferase), *pepX* (dipeptidyl aminopeptidase), *recG* (DNA recombinase), *rplB* (ribosomal protein 50S) and *uvrA* (exonuclease ABC). All these genes are included in the group of chromosomal genes. They were used in the analysis of the entire genome as well as in the comparative analysis carried out by Jabbari et al. creating a schematic allelic profile [56]. Researchers from the University in Wrocław carried out genotyping with multiple locations of these 8 genes in order to detect different types of *B. burgdorferi* in small mammals. All samples tested by means of this method were positive for *B. afzelii* [57]. The MLST method is very sensitive but also time-consuming and costly. This method is widely used in the population, evolutionary and phylogenetic studies. It allows the separation of *B. bavariensis* from *B. garini* strains. Therefore, it is considered an alternative method of *Borrelia* spirochaetes classification. It is also used in ecologic studies of these bacteria [36, 38].

## New Generation Sequencing (NGS)

In the last decade sequencing has identified the order of nucleotides in DNA now routinely used in molecular tests. In the case of a positive PCR result for *Borrelia* spirochaetes, verification is tested using the sequencing method. Several sequencing methods are used. Out of them, the most automatic and based on nanotechnology is the new generation method (NGS). Due to its use, the number of partially or fully described genome sequences of the *Borrelia* species is increasing in NCBI (National Center for Biotechnology Information). NGS is a very effective tool allowing the analysis of the entire microbiome, the transcriptome of tick-related pathogens [58].

## Conclusions

Lyme disease is currently one of the most commonly spread tick-related diseases in Poland. Each year the number of infected ticks, vectors transferring *Borrelia* spirochaetes, rises and as a result the number of infections among people has increased. The diagnosis of Lyme disease is difficult and often challenging. Methods for early detection of the disease are constantly updated. The most popular methods are serological and common among patients. Yet, they are not without difficulties. The field of molecular biology is particularly promising in the identification of *B. burgdorferi* as well as molecular differentiation used in the analysis of bacterial genomes. Molecular methods significantly shortened the diagnostic time for the DNA of *Borrelia* spirochaetes. In comparison to the conventional methods, they are characterized by high sensitivity, facilitated standardization and require a relatively small amount of test material. Action are directed at reducing the costs of these tests, decreasing prices of equipment and the agents used. An important aspect of molecular testing is highly qualified personnel.

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