PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 61 (2013), No 3-4

doi:10.3409/fb61_3-4.185

Rapid Identification of *Borrelia* by High Resolution Melting Analysis of the *groEL* Gene

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Accepted May 15, 2013

KOŚ W., WODECKA B., ANKLEWICZ M., SKOTARCZAK B. 2013. Rapid identification of *Borrelia* by high resolution melting analysis of the *groEL* gene. Folia Biologica (Kraków) **61**: 185-191.

This study examined the possibility of applying a new diagnostic method, high resolution analysis of DNA denaturation curve (high resolution melting – HRM), for identification of *Borrelia* species. DNA samples were obtained from *Ixodes ricinus* ticks collected from vegetation and removed from hunted roe deer. For differentiation of *Borrelia* species, the HRM protocol based on the analysis of the *groEL* gene was applied. A product characteristic for *Borrelia* was obtained in 19/123 samples (15.4%). The studied isolates were classified as four species: *B. garinii*, *B. valaisiana*, *B. afzelii* and *B. miyamotoi*. Two separate groups of isolates within the *B. afzelii* species were also found. The results show that the *groEL* gene is useful for rapid differentiation of *B. burgdorferi* sensu lato with the HRM method from different extracts of DNA and it also allows precise differentiation of *Borrelia* species and strains. The HRM method shortened and simplified detection and differentiation of *Borrelia* species from different biological sources.

Key words: *Borrelia*, PCR, HRM, molecular diagnosis

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Bacteria from the *Borrelia* genus belong to the order of spirochetes (*Spirochaetales*) and *Spirochaetaceae* family. Within this genus, there are two complexes: relapsing fever borreliae – RFB, including *B. hermsii* and *B. miyamotoi*, and Lyme disease borreliae – LD, which were defined as *B. burgdorferi sensu lato* (*s.l.*) (DERDAKOVA & LENCAKOVA 2005). The *B. burgdorferi s.l.* complex consists of at least 17 species (POSTIC *et al.* 2007, MARGOS *et al.* 2009, RUDENKO *et al.* 2009). The most common agents of human LB are *B. burgdorferi sensu stricto* (*s.s.*), *B. afzelii*, *B. garinii*, *B. lusitaniae*, and *B. spielmanii* (PIESMAN & GERN 2004, DE CARVALHO *et al.* 2008). Even though there are many applications of molecular methods for the diagnostics of Lyme borreliosis, their specificity and sensitivity are not satisfactory and they do not allow to differentiate among the *Borrelia* complexes mentioned above.

High resolution melting (HRM) is a relatively new method of DNA analysis introduced in 2002 (REED *et al.* 2007), as the easiest method of genotyping mutations and comparing the DNA sequences. This method is based on the denaturation of the DNA helix under the influence of temperature and it involves analysis of the product obtained with the traditional PCR method. The melting curves are determined on the basis of analysis of changes that occur in the fluorescence signal of the stain during this process (REED *et al.* 2007). Using the intercalating third generation stain in HRM enables precise analysis of the degree of denaturation of DNA. The advantage of the HRM method is high sensitivity allowing the recognition of DNA fragments differing by one pair of nucleotides. Moreover, controlled denaturation following earlier amplification of genetic material is carried out in the same test-tube. This reduces the risk of contamination of samples. Furthermore, electrophoresis is unnecessary, which significantly shortens the time of analysis of the amplified product (MONTGOMERY *et al.* 2007).

HRM analysis has been used for the rapid identification of many bacterial species (CHENG *et al.*

2006; FORTINI *et al.* 2007; JEFFERY *et al.* 2007; LIN *et al*. 2008, STEPHENS *et al.* 2008). Accordingly, the method seems to offer a new perspective in the analysis of DNA of *Borrelia* originating from blood, tissue biopsies or infected ticks, which are the vectors of these bacteria.

Molecular markers appropriate for HRM analysis in *Borrelia* were previously described for only three *Borrelia* species, i.e. *B. burgdorferi s.s*., *B. garinii* and *B. afzelii.* The following fragments of genes were used: *recA*, *p66* and *hbb* (PIETILÄ *et al.* 2000; MOMMERT *et al.* 2001; PORTNOÏ *et al.* 2006). In the present study we propose a new protocol allowing for the differentiation of *Borrelia* species belonging to RF and LD complexes using primers complementary to the *groEL* gene. In silico alignment of these genes and *groEL* revealed the possibility of differentiation of more *Borrelia* species using *groEL* than *recA*, *p66* or *hbb*. Therefore the aim of this study was to develop a sensitive, specific, and rapid HRM assay for the detection of *Borrelia* in various types of biological, especially human samples.

Material and Methods

The investigated material consisted of 123 isolates of DNA obtained from earlier studies carried out using the PCR-RFLP method (WODECKA 2007; SKOTARCZAK *et al.* 2008; WODECKA *et al.* 2010). Five of these isolates came from *Ixodes ricinus* ticks collected from vegetation, 61 from ticks derived from hunted European roe deer (*Capreolus capreolus*) and 57 from the blood of roe deer that lived in forests in the vicinity of Szczecin (Poland). Roe deer were shot during seasonal hunting; blood and ticks were collected immediately after death. Blood was taken from a heart chamber with the use of a syringe without needle, and directly mixed with 10% EDTA. The roe deer were adult males (exact age was not specified), shot in May-June 2007. Blood and ticks were collected from the same individuals. DNA from the blood of roe deer and from ticks collected from animals was isolated with the MasterPureTM DNA Purification Kit (Epicentre, Madison, WI, USA), and isolation of DNA from ticks collected from vegetation was carried out with ammonium hydroxide (WODECKA 2004).

PCR with high resolution melting analysis

A fragment of the *groEL* gene encoding a heat shock protein was used for the detection and differentiation of *B. burgdorferi s.l.* species. The primers gro-f4N (5'-TTCTACCAATAAAGAGAATATGAG-3') and gro-r1N (5'-TAAAACAAGAGCAGCAAGAGCATC-3') were developed for this study on the basis of conserved regions of groEL sequences of *Borrelia* derrived from GenBank using DNAMAN 5.2.9 software (Lynnon Biosoft, Canada). The primers were not complementary to *groEL* sequences of other spirochetes or tick-borne pathogens. The primers gave a PCR product of 181 bp length. The reaction mixture contained: 70 mM Tris-HCl (pH 8.6; 25°C), 16.6 mM $(NH_4)_2SO_4$, 0.75 mM of deoxyribonucleotide triphosphate (dNTP), 4 pmol of gro-f4N and gro-r1N primers, 3.5 mM $MgCl_2$, 0.5 U Allegro Taq DNA polimerase (Novazym, Poland), 0.4μl of EvaGreen stain (Jena Bioscience, Germany) and 1 μl of DNA suspension isolated from mammalian blood or collected ticks. The reaction conditions were as follows: preliminary denaturation at 95°C for 10 min., 40 cycles of denaturation at 95°C for 20 sec., primer annealing at 60°C for 35 sec. and elongation of the DNA chain at 72°C for 35 sec. The reaction was carried out in the RotorGene 6000 thermal cycler (Corbett Life Science, Australia). Control samples were made using 5 reference strains (Table 1): *B. garinii* (G23-07), *B. valaisiana* (BA9F9-05), *B. miyamotoi* (D110-07) and two *B. afzelii*. The latter species was differentiated at the level of strain as *B. afzelii* 1 (9W10-04) and *B. afzelii* 2 (ST28N7-05).

Controlled denaturation of DNA was performed at a temperature range between 75°C-90°C, with temperature increment of 0.1°C/sec. The analysis was carried out directly after standard PCR in the same reaction tubes and the results were saved as a file read by the Rotor-Gene_1_7_87 program for the usage of RotorGene 6000 thermal cycler (Corbett). The denaturation curves were normalized in order to enable their comparison and identification of genotypes on the basis of differences in temperatures of thawing with confidence level of 80%. The samples were compared with the reference isolates, the species affinity of which was previously assessed using the PCR-RFLP method (Table 1) and confirmed by sequencing of the *flaB* gene (WODECKA 2007; SKOTARCZAK *et al.* 2008; WODECKA *et al.* 2010).

Results

Analysis of DNA amplification products using the HRM method

An investigation using a 181 bp fragment of the *groEL* gene was carried out with regard to reference samples. The denaturation curves were subjected to normalization. Regions of normalization established in this study for the examined fragment of the *groEL* gene covered the temperature ranges from 77.07°C to 79.84°C and from 80.42 to 82.79°C.

Table 1

Reference strains used in the study

Borrelia species	Strain	Accession number[a]
B. garinii	G23-07	FJ518806
B. afzelii 1	9W10-04	FJ874924
B. afzelii 2	ST28N7-05	DQ650335
B. valaisiana	BA9F9-05	DQ650330
B. miyamotoi	D110-07	FJ518804

[a] flagellin gene.

Table 2

Comparison of detectability of DNA and *Borrelia* species identification in DNA samples from blood and from *I. ricinus* collected from vegetation and animals, by PCR-RFLP and HRM

Isolates	Method	n/+(%)	*B. garinii* n/+(%)	*B. valaisiana* n/+(%)	*B. miyamotoi* n/+(%)	*B. afzelii1* n/+(%)	*B. afzelii2* n/+(%)
I. ricinus/veg	HRM	5/4 (80)	–	–	–	2	2
	PCR-RFLP	5/5 (100)	–	–	1	4[a]	
I. ricinus/animal	HRM	61/12 (17)	2	1	3	–	6
	PCR-RFLP	61/12 (17)	3	–	3	6[a]	
Blood	HRM	57/3 (5)	1	–	–	2	–
	PCR-RFLP	57/3 (5)	2	–	–	1[a]	
Total	HRM	123/19 (15.4)	3 (15.8)	1 (5.3)	3 (15.8)	4 (21.1)	8 (42.1)
	PCR-RFLP	123/20 (16.3)	5 (25.0)	0 (0)	4 (20.0)	11[a] (55.0)	

[a] single type of *B. afzelii* was obtained in PCR-RFLP analysis.

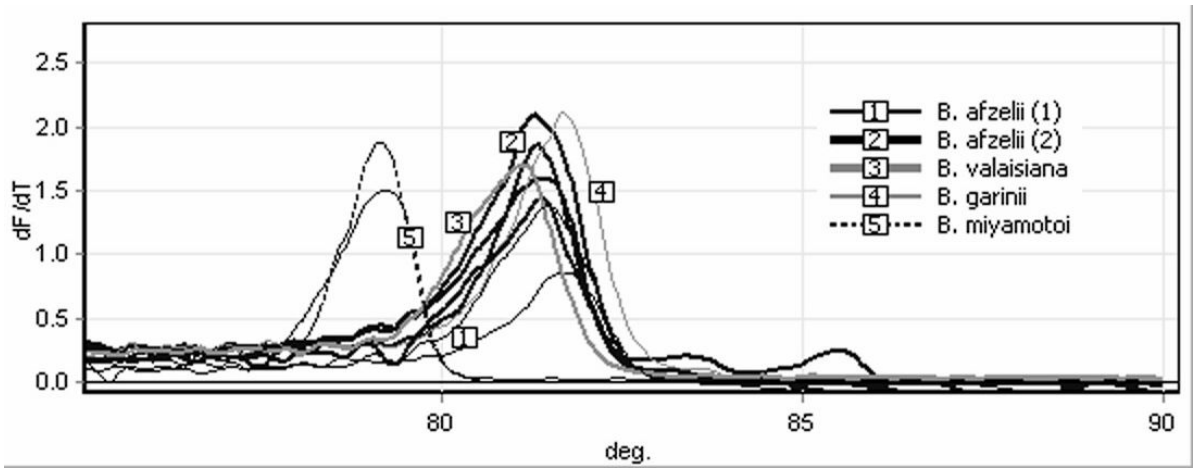

Fig. 1. Melting-points of amplification products of *groEL* genes of *Borrelia* species. *B. afzelii* type 1 (1), *B. afzelii* type 2 (2), *B. valaisiana* (3), *B garinii* (4), *B. miyamotoi* (5).

As a result of the analysis of 123 investigated isolates, 19 denaturation curves of PCR products were obtained, which showed genetic similarity to reference samples (Table 2). Denaturation curves characteristic for *B. garinii* species were found in 3 isolates, for *B. valaisiana* in 1, in 2 for *B. miyamotoi* and in 12 for *B. afzelii*, in which 4 isolates were qualified as *B. afzelii* 1, and 8 as *B. afzelii* 2 (Table 2).

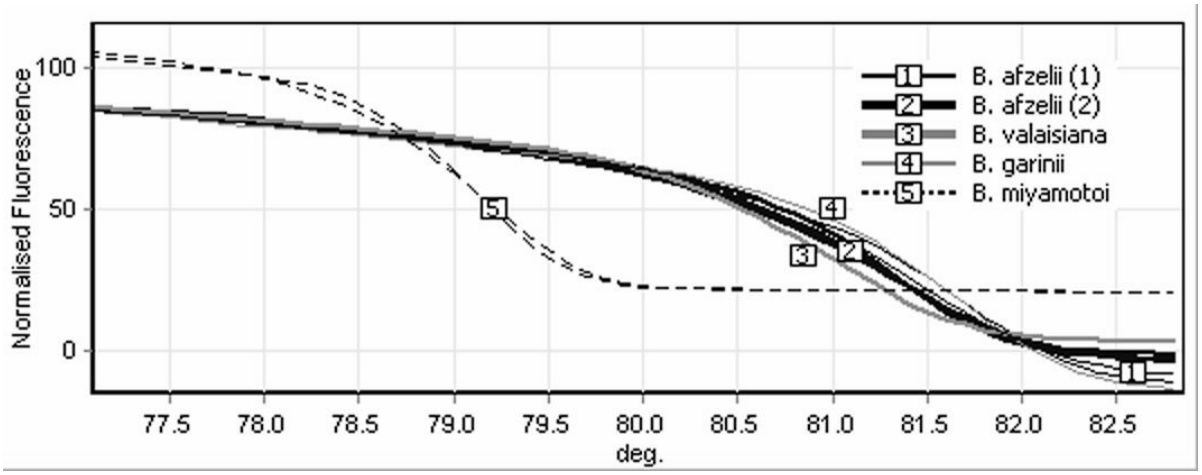

Fig. 2. Melting curves of the *groEL* gene fragment. *B. afzelii* type 1 (1), *B. afzelii* type 2 (2), *B. valaisiana* (3), *B garinii* (4), *B. miyamotoi* (5).

The denaturation curves obtained for *B. miyamotoi* were noticeably different from the curves obtained for *Borrelia* species belonging to the *B. burgdorferi s.l.* complex (Figs 1-3). Table 2 shows a comparison of detectability of DNA and *Borrelia* species identification in isolates of DNA from *I. ricinus* collected from vegetation, removed from animals and in blood isolates, carried out with the HRM method, and results of an earlier analysis using the PCR-RFLP method (WODECKA 2007; SKOTARCZAK *et al.* 2008; WODECKA *et al.* 2010).

Discussion

Borreliosis, known also as Lyme disease or tick-borne spirochetemia, is a serious health and epidemiological issue around the world, especially concerning people occupationally exposed to contact with ticks. More and more frequent cases of this disease have been reported in all regions of Poland (BILSKI 2009; BUCZEK *et al.* 2009; WODECKA *et al.* 2010). Although Lyme borreliosis has been widely recognized in Europe, diagnostic and therapy concepts are still a matter of discussion (STANEK & STRELE 2009). Even the introduction of molecular methods for the diagnostics of this disease has not altered this fact. Although detection of DNA of *B. burgdorferi s.l.* with PCR is increasingly used in laboratory diagnosis, this method has significant limitations (STANEK *et al.* 2011). One of the reasons is low sensitivity of PCR for detection of *B. burgdorferi s.l.* DNA in blood, plasma or serum samples from patients with Lyme disease. The low defer may be an indication of a low level of spirochetes in blood, lack of spirochetemia or passing spirochetemia (GOODMAN *et al.* 1995).

The aim of this study was to develop a sensitive, specific, and rapid HRM assay for the detection of *Borrelia* in various types of biological samples.

The efficiency of a PCR assay is determined by several factors. Among these, the selection of an appropriate gene target and primer set for PCR amplification are most important in development of any new PCR protocols (REED *et al.* 2007). In the present study, a key objective was to design selective primers that would allow amplification of all genes of spirochetes transferred by *I. ricinus* ticks. Accordingly, they should provide the possibility to amplify DNA of not only species belonging to *B. burgdorferi s.l.* complex but also DNA of *B. miyamotoi*, a species belonging to relapsing fever borreliae (RFB), while at the same time they should not amplify the genetic material of other bacteria (MERLJAK-SKOCIR *et al.* 2008). In the present study, for the amplification of DNA, we used primers complementary to the *groEL* gene. The whole length of this gene is 1600 bp, but only about 310 bp are well known for specific species and strains of *B. burgdorferi s.l.*, available in GenBank. Because of the high sensitivity of the HRM method in detecting small differences between investigated amplicons, analyzing short DNA fragments ranging from 100 bp to 200 bp and those having small variability is recommended, since the effectiveness of detecting differences is lower in conjunction with an increase of DNA chain (REED *et al.* 2007). At the same time, attention should be paid to marker selection in such a way that the differences between analyzed amplicons are not too large, because otherwise correct interpretation of the results may be affected (REED *et al.* 2007). Due to the high invariability of the *groEL* gene, its sequence, which encodes the 60-kDa heat shock protein GroEL, has already been used in genetic studies of these bacteria. The nucleotide sequences

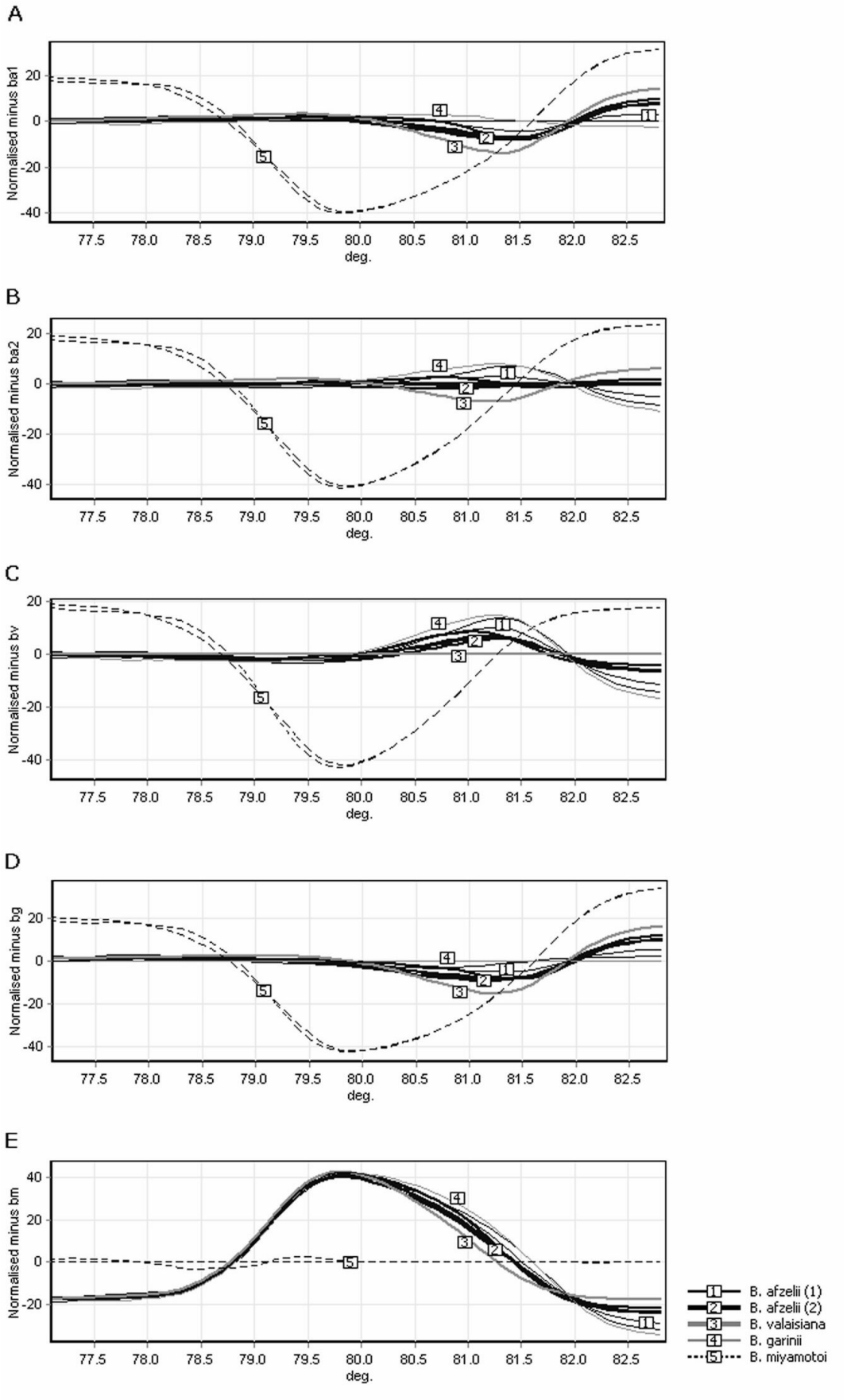

Fig. 3. Normalized graphs of denaturation curves of the *groEL* gene fragment: A – minus *B. afzelii* type 1, B – minus B. afzelii type 2, C – minus *B. valaisiana*, D – minus *B garinii*, E – minus *B. miyamotoi*.

(310 bp) of the *groEL* gene from 31 reference strains of *Borrelia* were determined and compared by LEE *et al.* (2003) and PARK *et al.* (2004). More than 92.3% similarity was observed among *B. burgdorferi s.l.* strains. In the phylogenetic trees constructed with the maximum-likelihood method (LEE *et al.* 2003) or with the unweighted pair group method (PARK *et al.* 2004), each species of *B. burgdorferi s.l.* was differentiated as a distinct entity. The usefulness of the *groEL* gene in differentiating relapsing fever group borreliae and establishing new species was shown by TOLEDO *et al.* (2010).

In the present work, the amplification of a fragment of the *groEL* gene with the PCR method, using primers gro-f4N and gro-r1N, resulted in a product whose molecular characteristics were consistent with the results obtained for the reference strains in 15.4% of studied samples. Samples analyzed with the HRM method were earlier investigated with the PCR-RFLP technique, and the level of detectability of *Borrelia* DNA was similar (16.3%, Table 2).

The highest percentage of effectiveness in the identification of species was obtained in samples originating from ticks collected from vegetation, while it was significantly lower in the case of isolates of *B. burgdorferi s.l.* DNA from ticks collected from roe deer and from isolates from roe deer blood (Table 2). The results show that the most stable product of the PCR reaction which may then be analyzed with the HRM method was obtained from unfed ticks collected from vegetation as in the case of an earlier analysis with the PCR-RFLP method. The presence of blood in engorged ticks may influence the detectability of *Borrelia* DNA therefore a more sophisticated method of DNA isolation, for example the phenol-chloroform protocol, may have to be used to improve the stability of the PCR product. Individual *Borrelia* species show diverse host specificity towards different species of tick hosts. Among these hosts, a significant role is played by the *Cervidae* family as a basic host group of adult ticks, which assures the continuity of their life cycle, yet it does not fulfil the condition of a competent reservoir of species from the *B. burgdorferi s.l.* complex (KURTENBACH *et al*. 2002; PIESMAN & GERN 2004; WODECKA 2007; SKOTARCZAK *et al.* 2008). The confirmation of this phenomenon and also the specificity and sensitivity of the HRM method is a low percentage of *Borrelia* infections in ticks collected from *Cervidae* obtained in this work as well as conformity of these results with those obtained earlier with the PCR-RFLP method (Table 2).

In earlier studies (WODECKA 2007; SKOTARCZAK *et al.* 2008; WODECKA 2008) we used a nested-PCR method based on the sequence of the *flaB* gene as the molecular marker for the detection of *Borrelia* DNA. Using two pairs of primers, i.e. outer: 132F and 905R, and inner: 220F and 823R, we studied isolates of DNA from the blood of roe deer, fed ticks removed from the roe deer and from unfed ticks collected from vegetation in forest localities around Szczecin. The restrictive analysis (PCR-RFLP) of amplicons obtained in these studies showed the affiliation of detected spirochetes to four *Borrelia* species, i.e. *B. garinii*, *B. afzelli*, *B. valaisiana* and *B. miyamotoi*. These species were also identified in the present work with the HRM method, so the results obtained with HRM demonstrated conformity with earlier studies carried out on the same material. The curves which were the effect of controlled denaturation of the *groEL* gene showed the autonomy of investigated species and similarity of isolates belonging to the same species (Fig. 3).

On the basis of different curves it was possible to separate two groups of isolates belonging to *B. afzelii* species as *B. afzelii* type 1 and *B. afzelii* type 2. The results show the possibility of using the *groEL* gene in the HRM method for the identification of not only species but also strains within known species of *B. burgdorferi s.l.* Such a solution increases the precision of analysis and enables differentiating species into strains having different levels of virulence.

Commonly used methods in the studies of isolates of *B. burgdorferi s.l.*, such as PCR-RFLP and nested-PCR require an electrophoretic division of reaction products in gel so that they can be visualized, which increases the risk of contamination, lengthens the duration of a study and raises costs (CHENG *et al.* 2006). When fluorescently – labelled probes and real-time PCR are not required, high-resolution methods offer cost and simplicity advantages over other closed-tube genotyping approaches. Furthermore, cost advantages of HRM derive from the simplicity of the technique (REED *et al.* 2007; SLINGER *et al.* 2007). Obtained results are easy to interpret and HRM, used in an appropriate way, may replace sequencing in 95-99% (REED *et al.* 2007), also significantly decreasing the cost of studies.

The application of controlled and high resolution analysis of denaturation for the detection and differentiation of species and strains of *B. burgdorferi s.l.* provides a new opportunity in the diagnostics of Lyme borreliosis. However, the growing number of DNA sequences of *Borrelia* submitted to GenBank annually indicate that there is a need to validate the proposed method to establish the true variability of the *groEL* gene within different *Borrelia* species and between them in order to avoid the risk that the same melting curves may be obtained for different DNA sequences. Therefore studies applying the proposed protocol

using more samples with consideration of different DNA isolation protocols are planned.

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