Part 1. Veterinary medicine

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INDICATION AND SPECIES DIFFERENTIATION OF THE *BABESIA* PROTOZOAN GENUS BY THE POLYMERASE CHAIN REACTION

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Summary. According to the information obtained from numerous literature sources, the *Babesia* protozoan genus is known to be the most thoroughly studied as to the structure of the gene encoding 18S of ribosomal RNA. Based on the information provided by the international database of DNA sequencing 'GenBank', conservative and polymorphic sites of the above gene's nucleotide sequences belonging to different *Babesia* species have been defined using MEGA4 software.

The conservative 18S gene region of rRNA has been used to develop the system of oligonucleotide primers allowed to indicate DNA of six representatives *of Babesia* species. Variable regions of this gene were used to develop oligonucleotide primers for *Babesia canis*, *Babesia bovis*, and *Babesia divergens* representatives DNA identification on species-specific level.

The multiplex PCR diagnostic test system has been developed for determining presence of DNA of the representatives of six *Babesia* genus species (*B. canis*, *B. divergens*, *B. caballi*, *B. major*, *B. bigemina*, *B. bovis*) in any biological sample.

The research results of biological samples confirmed the sensitivity and analytical specificity of the developed PCR test system for the diagnostics and differentiation of the certain *Babesia* genus species.

Keywords: *Babesia,* multiplex PCR, test system, oligonucleotide primers, indication, differentiation

Introduction. Babesioses is a group of seasonal transmissible diseases of mammals belonging to different species, caused by protozoa of *Babesia* genus. These pathogens are among the most common haemosporidial parasite of mammals (Boustani and Gelfand, 1996).

In veterinary practice, animal babesioses are mainly diagnosed in accordance with etiological factors (season, presence of ticks-carriers on the animal, etc.), clinical manifestations and microscopy of stained peripheral blood preparation to detect babesia in erythrocytes (CLSI, 2000). However, these methods are sufficiently reliable only in cases of significantly developed typical clinical manifestation of the disease, while at early stages or at atypical form of the disease of the invasion, they are ineffective. Taking into account serious consequences at already developed pathological process and sufficiently high toxicity of chemotherapeutic drugs, the problem of early, highly sensitive and specific diagnostics is very important.

Currently, there are different methods of laboratory diagnostics. The methods with high specificity and sensitivity are, above all, molecular genetics, including PCR. Today, there are PCR test kits to determine *Babesia* spp., as well as for separate species differentiation: *Babesia microti, Babesia divergens, Babesia canis*, etc. (Peleg et al., 2009; Adaszek and Winiarczyk, 2010; Müller et al., 2010). However, the use of Western diagnostic agents requires significant financial resources, therefore we have acquired Russian PCR test system produced by 'GenPak' company to indicate *Babesia* spp., which proved to be ineffective due to its lack of sensitivity.

The aim of the study. Based on the above-mentioned facts, we have set up the goal to develop *in-house* PCR test system for laboratory diagnostics of the animal babesioses and differentiating some of their pathogens by species.

Materials and methods. The study was performed at the Genetics laboratory of Institute for Pig Breeding and Agroindustrial Production of NAAS.

Databases of nucleotide sequences GenBank (USA) were used to obtain the nucleotide sequences of the gene encoding 18S of rRNA in nine species of *Babesia* genus protozoa which are pathogenic for different species of mammals (*B. bovis, B. caballi, B. canis, B. divergens, B. equi, B. felis, B. gibsoni, B. major,* and *B. microti*) for the purpose of the primer design.

The primary nucleotide sequences of the gene encoding 18S of rRNA in the above nine species

of *Babesia* genus protozoa were aligned using MEGA4 software (Tamura et al., 2007). Conservative and polymorphic for different species sites were selected to design oligonucleotide primers.

Using the FastPCR software, oligonucleotide primers sequences were obtained together with their annealing temperature ranges (Kalendar, Lee, and Schulman, 2014). Among the obtained primers designs two upstream and three reverse oligonucleotide primers were selected to provide indication of six *Babesia* species with simultaneous differentiation of the three of them, which mainly circulate in Ukraine.

Synthesis of oligonucleotide primers were ordered in the Thermo Electron Corp. (Germany) according to the developed designs.

The synthesized primers were diluted with sterile double-distilled deionized water to the stock concentration of 100 pM/μl, and then to the operating concentration of 20 pM/μl.

Besides the primers, PCR reagents manufactured by Fermentas UAB (Lithuania) were used in the test systems, namely: deionized water, PCR buffer, $MgCl_2$, deoxyribonucleoside triphosphate (dNTP) solution and *Taq*-polymerase.

Polymerase chain reaction was performed in 0.6 ml polypropylene microcentrifuge tubes in the thermocycler 'Tertsic 2' produced by 'DNA technology' company (Russian Federation) in 25 μl of PCR mixture by using the in-house system of oligonucleotide primers flanking the fragments of 18S gene of rRNA in *Babesia* genus protozoa.

DNA isolation from the studied biological samples was performed by the sorption method using the commercially available kit 'DNA-sorb-B' produced by 'AmpliSenS biotechnologies' (Russian Federation).

Blood samples from horses, dogs, and cattle with babesiosis and suspensions of in saline pounded in a mortar ticks were used for the development of PCR test systems. *Babesia* invasion level in the samples was determined by the microscopic study of the peripheral blood preparations stained by Romanovsky using Romanovsky-Giemsa stain (Gill, 2013).

Results and discussion. Designing of the oligonucleotide primers for *Babesia* genus-specific detection by polymerase chain reaction required search for both conservative and polymorphic regions of 18S rRNA gene in *B. canis, B. caballi, B. divergens, B. major, B. bigemina*, and B. bovis, which are found neither in any other protozoa nor microorganisms and suitable for electrophoretic detection (flanking the DNA fragment with appropriate length). The primary 18S rRNA gene sequences of the *Babesia* genus representatives were used for alignment. Following access codes sequences were downloaded from GenBank database: *B. canis* (FJ200218, EU622793, EU165369, EU711061), *B. gibsoni* (FJ55453), *B. major* (EU622907), *B. felis* (AF244912), *B. divergens* (EU182595, DQ866843, DQ866844), *B. microti* (FJ480420), *B. bovis* (EU407240, FJ426364, EF458215, EF458214, EF643475, EF643473, EF643466, AY150059, L19078), *B. caballi* (EU888901, EU642514), and *B. equi* (AY150063, Z15105, DQ287951).

As a result of the primary sequences alignment both conservative and polymorphic nucleotide sequences for six babesiosis agents' species were identified and became the basis for oligonucleotide primers designed for the multiplex PCR test systems.

Using the FastPCR software, the oligonucleotides were designed together with the recommended annealing temperatures.

Among nine designed primers five were selected for using in the multiplex PCR. Thus, the developed system consists of two upstream (forward) primers: BCANF: 5'-GTGACCCAAACCCTCACCAGA-3'; BSPF: 5'-CCATTGGAGGGCAAGTCTGGT-3', and the three downstream (reverse) ones: BDIVR: 5'-TCCCAAAAGCGAAGTGCAATCTCG-3'; BBOVR: 5'-CCAAAGTCAACCAACGGTACGACA-3'; BSPR: 5'-ACGAATGCCCCCAACCGTT-3'.

The reduction of the number of the oligonucleotide primers required for the multiplex PCR test system is important to prevent their overload respectively.

Amplified products in PCR with listed primer set contained fragments of 18S rRNA gene of the *Babesia* genus representatives, sizing from 268 to 298 bp, conservative for babesia of six species (*B. canis, B. caballi, B. divergens, B. major, B. bigemina*, and *B. bovis*) that causes the disease in dogs, horses, cattle and humans (Table 1), as well as fragments sizing 325, 233 and 146 bp, specific for babesia of three species (*B. canis, B. bovis, B. divergens*), which are mainly circulating in Ukraine (Lets et al., 2015; Maidannyk, 2013; Prus, 2006; Prus and Peryn, 2013) (Table 2).

The logical diagram of the primers system and amplicon's size flanked by them is presented in Fig. 1.

Optimization of the PCR conditions included adjustment of the reaction mixture and amplification temperature ranges.

As a result of PCR protocol agreement, the best parameters of the reaction mixture were following: 2.5 μl of 10-fold buffer (670 mM of Tris-HCl, pH 8,8 at 25 °C, 20 mM of BSA, 166 mM of ammonium sulfate $(NH_4)_2SO_4$, 100 mM of 2-β-mercaptoethanol) (Fermentas UAB, Lithuania), 2.5 μl of 2.5 mM of dNTP (Fermentas UAB, Lithuania), 2 μl of 50 mM of $MgCl_2$ (Fermentas UAB, Lithuania), 2–3 units

of *Taq*-polymerase (*Thermus aquaticus*) (Fermentas UAB, Lithuania), 0.5 µl (0.1 opt. unit) of each of the five primers and the studied DNA sample 1 µl (20 µg/ml). Deionized water was added to the volume of 25 µl. The amplification mixture was covered with a layer of 25 µl of mineral oil.

Table 1 – The amplicon's size of six representatives of *Babesia* genus, obtained using the primers pair BSPF/BSPR (*Babesia* sp.)

No.	Agent species	PCR product size
1	Babesia canis	298 bp
2	Babesia divergens	298 bp
3	Babesia caballi	287 bp
4	Babesia major	287 bp
5	Babesia bigemina	284 bp
6	Babesia bovis	268 bp

Table 2 – Primers pairs and PCR products' lengths in different *Babesia* species representatives

No.	Primers pair	DNA amplified	PCR product size	Notes
1	BCANF/BSPR	Babesia canis	325 bp	unique
2	BSPF/BSPR	*Babesia* sp.	299–285–268 bp	common for 6 species
3	BSPF/BBOVR	Babesia bovis	233 bp	unique
4	BSPF/BDIVR	Babesia divergens	146 bp	unique

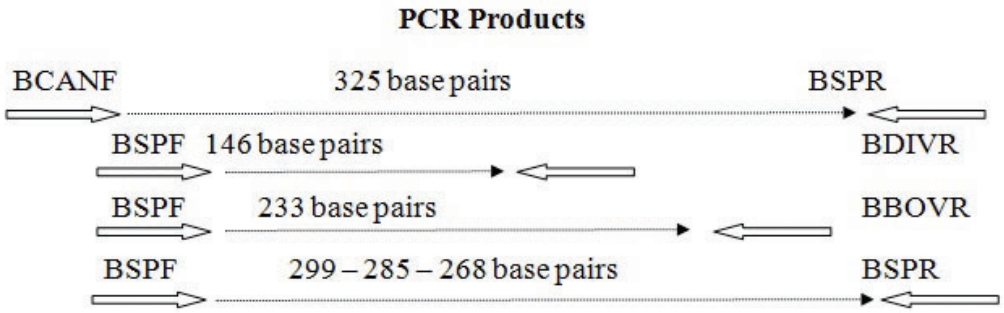

Figure 1. Diagram of multiplex PCR test system amplicon's size

The best amplification parameters made:

93 °C..... 60 sec.
93 °C..... 30 sec.
60 °C..... 30 sec.
72 °C..... 30 sec.
72 °C..... 60 sec.
} 35 cycles

Analytical specificity testing for the developed test system was the next step after adjustment of the PCR parameters.

The following eight biological materials were tested: the DNA sample isolated from the tick removed from a babesiosis-infected dog and pounded in a mortar — No. 1; DNA sample isolated from peripheral blood of a babesiosis infected dog with low parasitemia level (1%) — No. 2; DNA sample isolated from peripheral blood of a babesiosis-infected dog with high parasitemia level (5%) — No. 3; DNA sample isolated from the blood of a gnotobiotic puppy experimentally infected with babesia (parasitemia level 1%) — No. 4; DNA sample isolated from the blood of gnotobiotic puppy from the control group — No. 5; DNA sample isolated from the blood of a babesia infected horse (parasitemia level 2%) — No. 6; DNA sample isolated from the blood of a babesia-infected heifer (parasitemia level 1%) — No. 7; DNA sample isolated from the blood of an infected cow (parasitemia level 1%) — No. 8.

These biological material samples were selected based on the fact that seven of them (No. 1–4, 6–8) credibly contained DNA of one of babesiosis agents' species, and one of them (No. 5), collected from the gnotobiotic puppy of the control group was free from any of them.

Two bands (325 bp and 298 bp) correspond to the DNA fragments of the 18S rRNA gene of *Babesia canis* and to the one indicating the *Babesia* genus were detected in tracks 1–4; in the 5th track there were no bands; in the 6th track there was detected the 287 bp amplicon, that by its electrophoretic mobility corresponds to the DNA fragment of the 18S rRNA gene of *Babesia* genus protozoa; in the seventh track there are bands (233 bp and 270 bp) that in their

electrophoretic mobility correspond to the DNA fragments of the 18S rRNA gene of *Babesia bovis* and the one that indicates the *Babesia* genus; in the eighth track there are bands (146 bp and 298 bp) that in their electrophoretic mobility correspond to the DNA fragments of the 18S rRNA gene of *Babesia divergens* and the one that indicates the *Babesia* genus (Fig. 2). No amplification products were detected in the negative control track.

The next step was to study both the materials (containing babesia and babesia free), as well as materials containing *Chlamydia* and *Leptospira* in three repeats with different amounts of the studied DNA. It should be noted that intensity of luminescence bands at introducing 2 µl (20 µg/ml) and 1 µl (20 µg/ml) of DNA on the electrophoregram into the reaction mixture did not differ visually, while at introducing 0.5 µl (20 µg/ml) of DNA, luminescence bands was evidently weaker, and some bands were almost invisible at all. The results of the study are presented in Table 3.

Thus, the results of studying the above biological samples by the PCR method demonstrated analytical specificity and sensitivity of the developed multiplex test system.

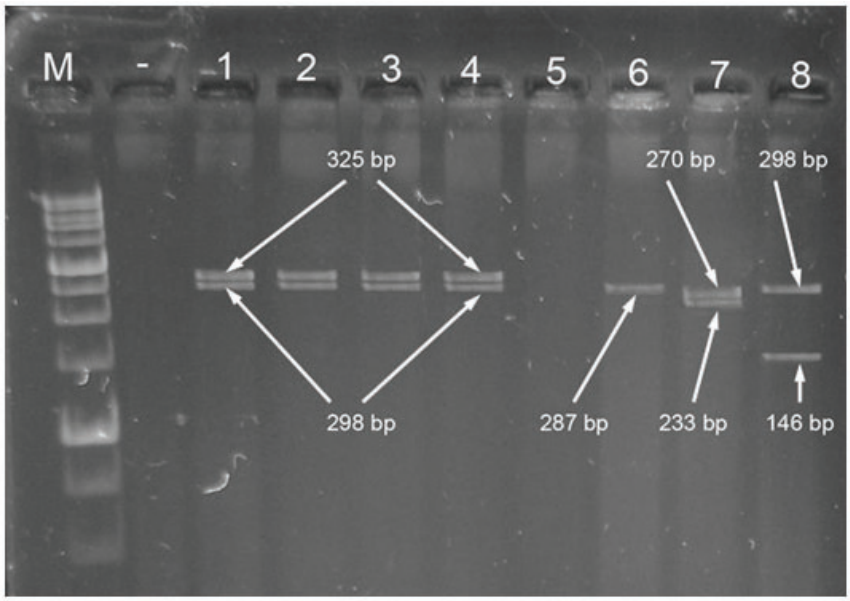

Figure 2. Electrophoregram of PCR-products of the DNA samples from biological material tested by multiplexed test system for indication and species differentiation of the *Babesia* genus, namely DNA samples isolated from the tick removed from a babesiosis infected dog, from the peripheral blood of babesiosis infected dogs, from blood of gnotobiotic cubs of the experimental and control groups, from blood of a babesia-infected horse, a heifer and a cow. Tracks: M — 100 bp DNA size marker *pUC19/MspI* (Fermentas UAB, Lithuania); «–» — negative control; 1–4 — 325 bp (*B. canis*) and 298 bp (*Babesia* genus) bands; 5 — negative result; 6 — 287 bp (*Babesia* genus) band; 7 — 270 bp (*Babesia* genus) and 233 bp (*B. bovis*) bands; 8 — 298 bp (*Babesia* genus) and 146 bp (*B. divergens*) bands

Table 3 – Testing results of the multiplex PCR test system developed to indicate protozoa of Babesia genus as to its analytical specificity and sensitivity

No.	Type of the sample studied	Study result										
		DNA amount in the reaction mixture									Band size	Agent species
		2 µl (20 µg/ml)			1 µl (20 µg/ml)			0.5 µl (20 µg/ml)				
		1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat	1 repeat	2 repeat	3 repeat		
1	tick removed from a babesiosis infected dog	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
2	tick removed from a babesiosis infected dog	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
3	tick removed from a babesiosis infected horse	+	+	+	+	+	+	+	+	+	287	*Babesia* spp.
4	tick removed from a babesiosis infected cow	+	+	+	+	+	+	+	+	+	146, 298	*Babesia divergens*
5	tick obtained from the natural environment	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
6	tick obtained from the natural environment	–	–	–	–	–	–	–	–	–	–	–
7	tick obtained from the natural environment	+	+	+	+	+	+	+	+	+	287	*Babesia* spp.
8	blood of a babesiosis infected dog	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
9	blood of a babesiosis infected dog	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
10	blood of a babesiosis infected dog	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
11	blood of a healthy dog	–	–	–	–	–	–	–	–	–	–	–
12	blood of a gnotobiotic cub infected with babesia	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
13	blood of a gnotobiotic cub infected with babesia	+	+	+	+	+	+	+	+	+	298, 325	*Babesia canis*
14	blood of a gnotobiotic cub from the control group	–	–	–	–	–	–	–	–	–	–	–

15	blood of a babesiosis infected horse	+	+	+	+	+	+	+	+	+	287	*Babesia* spp.
16	blood of an anaplasmosis infected horse	–	–	–	–	–	–	–	–	–	–	–
17	blood of a healthy horse	–	–	–	–	–	–	–	–	–	–	–
18	blood of a babesiosis infected cow	+	+	+	+	+	+	+	+	+	146, 298	*Babesia divergens*
19	blood of a babesiosis infected heifer	+	+	+	+	+	+	+	+	+	233, 270	*Babesia bovis*
20	blood of a healthy cow	–	–	–	–	–	–	–	–	–	–	–
21	control *Clamydia abortus* DNA	–	–	–	–	–	–	–	–	–	–	–
22	control *Clamydia pecorum* DNA	–	–	–	–	–	–	–	–	–	–	–
23	control *Clamydia psittaci* DNA	–	–	–	–	–	–	–	–	–	–	–
24	Leptospira (LSU strain, *Louisiana* serovar, *Louisiana* serogroup)	–	–	–	–	–	–	–	–	–	–	–
25	Leptospira (493 *Poland* strain, *polonica* serovar, *Sejroe* serogroup)	–	–	–	–	–	–	–	–	–	–	–
26	Leptospira (*Hond Utrecht IV* strain, *canicola* serovar, *Canicola* serogroup)	–	–	–	–	–	–	–	–	–	–	–

Conclusions. The developed multiplex PCR test system consists of two upstream (forward) and three downstream (reverse) oligonucleotide primers flanking the different sized DNA fragments of the gene encoding 18S rRNA of *Babesia* genus protozoa, permits to detect the Babesia DNA in any biological material and to differentiate species belonging of the most common three agents of this invasion in Ukraine. Indication and species differentiation of the *Babesia* genus representatives is provided by the visual assessment of the amplified fragments by different sizes of bands in the electrophoregram in 2.0% agarose gel.

Approbation of the developed PCR test system testifies to its sensitivity and specificity.

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