

Analysis of the amino acid sequences of 18S RNA strains of *Babesia canis* isolated from dogs, including the analysis of serum proteome of protozoa infected dogs

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ABSTRACT. The aim of this paper was to analyse the amino acid sequences of the 18S rRNA gene of *Babesia canis* strains and the proteomic analysis of the serum of dogs infected with three various genotypes: 18S rRNA *B. canis*. Material for the research was DNA *B. canis* obtained from dogs with babesiosis. In total, 60 DNA tested samples were divided into three groups (20 samples each). The groups were formed by DNA samples of the sequences marked as 18S RNA-A (group 1), 18S RNA-B (group 2), and 18S RNA-C (group 3). The basis for the classification of protozoa to a specific group was the location of relevant nucleotides (GA, AG, or TT) in position 150-151 of the tested nucleotide sequence 18S rRNA. Nucleotide sequences were transcribed into amino acid sequences and then analysed using DNASTAR software. From all 60 infected and ten healthy dogs (control group), the serum was taken to make proteomic tests using MALDI-TOF mass spectrometer. It was demonstrated that the mutations found in position 150 and 151 of the nucleotide sequence, result in a change of amino acid sequences. Moreover, it was also demonstrated that the disease course in dogs infected with different strains of protozoa is different. Each of the analysed strains of protozoa induced in the serum of infected animals the appearance of a protein fraction of mass 51 kDa, which may then be treated as a nonspecific disease marker used for the diagnosis of this disease but not to differentiate the protozoa strains.

Key words: *Babesia canis*, 18S rRNA, PCR, dog, MALDI-TOF

Introduction

Canine babesiosis is a common and clinically significant tick-borne disease caused by hematozoan parasites of the genus *Babesia* [1]. The classification of *Babesia* spp. places them in the order Piroplasmida within the phylum Apicomplexa. Two morphologically distinct forms of the erythrocytic stage in the canine host were recognized in early studies that led to the naming of the larger form, measuring approximately 3–5 µm as *B. canis*, and the smaller (1–3 µm) as *B. gibsoni*. On the basis of cross-immunity, serological testing, vector specificity and molecular phylogeny *Babesia canis* was reclassified into three sub-species: *B. canis*, *B. rossi* and *B. vogeli* and unnamed yet large *Babesia* detected in

dogs in North America. All of them are now considered to be separate species [2,3].

Clinically, all these pathogens cause remittent fever, progressive anaemia, haemoglobinuria, and marked splenomegaly and hepatomegaly in dogs and, in some cases, the death of infected animals [4,5]. In Poland, only the presence of *B. canis* has been noticed. The genetic analysis of Polish isolates of *B. canis* helped to distinguish three genotypes within this species: 18S rRNA-A and 18S rRNA-B (GenBank accession numbers: EU622792 and EU622793, respectively), and the new as yet unnamed 18S rRNA-C strain responsible for subclinical babesiosis [6,7]. However, no analysis of the amino acid sequence of gene 18S rRNA from the isolates of *B. canis* causing the disease in dogs has

been made so far and it has not been established yet if the changes in nucleotide sequence induced a change in the amino acid sequence. Additionally, Adaszek et al. [8] demonstrated, using MALDI-TOF mass spectrometer, the presence of the protein fraction of mass ca. 52 kDa in the blood serum of dogs infected with protozoa. However, he did not define if its presence in the serum of sick animals was correlated with one specific strain of *B. canis*, or if it appeared as a result of an invasion of the all three genotypes of 18S rRNA that were found in domestic dogs.

The aim of this paper was to analyse the amino acid sequences of the 18S rRNA gene of *B. canis* strains and to conduct the proteomic analysis of serum of the dogs infected with three various genotypes of the 18S rRNA *B. canis*.

Materials and Methods

DNA samples. The material for the research was the DNA of *B. canis* obtained from dogs with babesiosis. The disease was recognized on the basis of PCR results using starters: BAB GF2 and BAB GR2 that amplify the fragment of gene 18S rRNA of length 559 bp [6]. In total, 60 DNA tested samples were divided into three groups (20 samples each). The first group included the DNA samples of the sequence marked as 1 (18S RNA-A) originating from dogs with light babesiosis; the second group included the DNA samples of the sequence marked as 2 (18S rRNA-B) originating from sick dogs with acute babesiosis; the third group included the DNA samples of the sequence marked as 3 (18S rRNA-C) obtained from dogs with subclinical babesiosis [7,9].

All nucleotide sequences of group 1 had guanine in position 150 and adenine in position 151, whereas in the sequences of group 2 were adenine in position 150 and guanine in position 151. All *Babesia* sequences included into group 3 had thymine in both positions 150 and 151 (Table 1). The remaining nucleotide sequence of the analysed gene fragment of 18S rRNA in all three groups was identical.

Analysis of amino acid sequences of gene 18S rRNA *B. canis*. Nucleotide sequences were transferred into the amino acid sequences and were then analysed using DNASTAR software (Madison, WI, USA). A phylogenetic analysis was conducted in PHYLIP 3.5 software [10]. Dendrograms were constructed according to the Fitch-Margoliash method.

MALDI-TOF MS. The blood of all 60 dogs infected with the tested protozoa was sampled and additionally the blood of ten healthy control dog individuals were taken for proteomic tests using MALDI-TOF mass spectrometer. Whole blood samples were collected from all animals into test tubes containing a coagulation accelerator, which were then centrifuged to obtain serum that was used for proteomic testing.

Serum samples of 50 µl were vortexed, diluted tenfold and then cleansed on 0.2 µl Zip-Tip microcolumns (Merck Chemicals) according to a standard procedure (TN 226) that included preliminary activation of the stationary phase with H₂O:ACN solutions (Merck Chemicals). The prepared serums were mixed with the SA matrix (sinapinic acid) suspended in a TA 30 solution 30 (70:30 0.1% TFA in H₂O:ACN). A layer of the SA matrix suspended in EtOH HPLC Grade (Merck Chemicals) was placed on a MTP Polished Steel holder (Bruker). After the matrix dried, the test samples were placed on analytical spots (50:50 sample: SA in TA 30). Three analyses with the Ultraflex extreme mass spectrometer (Bruker) were performed for each sample within the weight range of 20 to 100 kDa. The spectrometric analysis was conducted using the flex Control 3.3 (build 108) programme, while the spectra were analysed with the flexAnalysis 3.3 (build 80) programme [8].

Results

Results of the analysis of amino acid sequence of the fragment of gene 18S rRNA

Computer translation of nucleotide sequences enabled the demonstration of amino acid changes in protein coded by gen 18S rRNA between the individual groups of *B. canis*. As the point of reference there was the sequence of isolates of group 1:

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VL.LE.W.PKPSPE.QLEGKSGASSRGNSSNSV
Y.TCCS.KARSCIFALAV.PFGWLFRRFRWEFPP
LL.EN.SVSSRLLS.ILQHGIIIE.DFGSILLVIEP.
WLIGTVGGIRI.LSEVKFLDLLKTNYESIC QG
RFH.SRTKVRGSKTIRYRRSPNHKLCRLVIGG
RRFLTPSGT.EKSKSLG
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It was demonstrated that the mutations found in position 150 and 151 of the nucleotide sequence, consisting in the conversion of guanine into adenine and adenine into guanine (group 2) as well as guanine and adenine into thymine (group 3), transferred into a change of amino acid sequences.

Table 1. Differences in nucleotide sequences in analysed rRNA fragment between the tested groups 18S RNA (1-3) of *B. canis*

Group	Position of nucleotide	Nucleotide change	Codon change
1	Lack	Lack	Lack
2	150	G → A	TTG → TTA
	151	A → G	ACG → GCG
3	150	G → T	TTG → TTT
	151	A → T	ACG → TCG

Inasmuch as in group 2, the replacement of nucleotides in position 150 of the tested gene segment proved to be a polymorphism or silent mutation, in the case of which the replacement of the codon TTG → TTA did not induce the conversion into the coded amino acid (leucine), the replacement of adenine into guanine in position 151 caused the change in codon ACG → GCG, which resulted in the replacement of alanine into threonine (Table 2).

Table 2. Differences in amino acids sequences in analysed rRNA fragment between the tested groups 18S RNA *B. canis*

Group	Position of amino acid	Amino acid change
1	Lack	Lack
2	50	Leu → Leu
	51	Ala → Thr
3	50	Leu → Phe
	51	Ala → Ser

In the case of group 3, both nucleotide mutations in positions 150 and 151 resulted in the replacement of coded amino acids from leucine into phenylalanine and from alanine into serine (Table 1).

Results of proteomic analysis

Proteomic analysis demonstrated the presence of eight protein fractions ranging from 20 to 100 kDa in serum samples obtained from all the tested animals, in the studied group and in the control group. Moreover, an additional protein fraction of approximately 51-52 kDa was found in all the serum samples obtained from the dogs with babesiosis regardless of the fact that the samples belonged to group 18S rRNA-A, 18S rRNA-B or 18S rRNA-C. These proteins were not found in the

serum of any of the control group dogs. The analysis of mass spectrograms of tested serum samples and the comparison of the control group and the test group spectra did not reveal any other significant differences.

Discussion

In recent years the range of occurrence of canine babesiosis in Poland has been seen to be extending. The disease is diagnosed increasingly, almost every year, and its course has become more and more diverse. Until recently a primary symptom of babesiosis in dogs in Poland was a colour change of urine and renal failure as a main complication [9]. Currently, in animals with babesiosis you may observe gastrointestinal abnormalities, disorders of respiratory system, nervous system or even motor system, MODS or SIRS are possible complications [9,11].

In earlier studies the relationship between virulence of *B. canis* and the differences in protozoa genome and their belongingness to group 18S rRNA-A, 18S rRNA-B and 18S rRNA C was demonstrated [6,9,12]. It might be surprising that the differences in the nucleotide sequence of gene 18S rRNA concerned only two nucleotides in position 150 and 151 of the tested gene fragment, but their consequence was a change in an amino acid sequence of the coded protein, which was the cause of differences in the clinical course of the disease. In all dogs infected with strains 18S rRNA-A, the course of disease was mild, in animals infected with strains 18S rRNA-B it was acute, while in dogs infected with strains 18S rRNA-C, it run subclinically. The data indicate that even such a slight change as point mutation in the conservative area of the genome of the parasite is reflected in the clinical image of the disease in dogs.

Each of the analysed protozoa strains induced

the appearance of protein fraction of mass 51 kDa in the serum of infected animals. The protein fraction of similar weight – 51-52 kDa was observed in own studies on the serum of *B. canis*-infected dogs: it was found in SPA antigen obtained from the supernatant of the protozoa *in vitro* culture [13]. The fraction appeared to be strongly immunogenic, which was confirmed in Western blot tests by Adaszek et al. [13]. It was the protein of 51-52 kDa that induced the strongest reaction with serum samples obtained from the dogs vaccinated with SPA [13,14]. Considering the above, it may be inferred that these proteins are released into a dog's serum by the protozoa after infection and they result in a signal in the infected dogs' serum which may then be treated as a nonspecific disease marker used naturally for the diagnosis of this disease, but not to differentiation of the protozoa strains. Own observations indicate that MALDI-TOF MS is a sensitive and a reliable diagnostic technique. The test results achieved were 100% compliant with molecular (PCR) testing.

The studies on the structure of genetic and amino acid structure of protozoa *Babesia* and serum proteomic tests of the blood serum of the animals infected with those protozoa appear to be crucial for understanding the differences in the course and the recognition of the pathogenesis of this disease, and also for both the diagnosis of babesiosis and the development of methods of specific prophylaxis. The research so far on proteome *B. canis* are very fragmentary [15,16]. There is only one literature report available regarding the use of mass spectrometry in testing protein profiles of *B. canis*-infected dog serum. The results obtained by the authors indicated that the technique may be used to monitor the clinical course of babesiosis and to study the pathogenesis of the disease [17]. Therefore, further research in this field appears to be absolutely justified.

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