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Original papers

Specificity of mass spectrometry (MALDI-TOF) in the diagnosis of *Babesia canis* regarding to other canine vector-borne diseases

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ABSTRACT. The canine vector-borne diseases (CVBD) is a term, which describes a range of infectious or/and parasitic diseases whose etiological agents are transmitted by vectors. CVBD are becoming more widely in the world in relation to global warming and the increasing number of infected vectors. The aim of this study was to assess rapid mass spectrometry (MS) – based proteomics analyses for diagnosis of *Babesia canis*, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* infections in dogs. The study was conducted on four groups of dogs – healthy dogs (group 1, n=10) and dogs infected with *B. canis* (group 2, n=20), *A. phagocytophilum* (group 3, n=20) and *B. burgdorferi* (group 4, n=20) which demonstrated symptoms of the diseases. The MALDI-TOF (Matrix Assisted Laser Desorption Ionization with Time of Flight detector) MS technique revealed the presence of specific protein fraction of 51–52 kDa only in the blood serum of all the animals infected with the *B. canis* protozoa. The proteins are suspected to be disease markers, whereas the MALDI-TOF technique itself has high specificity and sensitivity and can be applied in the diagnosis of canine babesiosis.

Key words: *Anaplasma phagocytophilum*, *Babesia canis*, *Borrelia burgdorferi*, MALDI-TOF, proteomics

Introduction

The term canine vector-borne diseases (CVBD) includes a wide variety of diseases of infectious or parasitic aetiology whose agents are transmitted by ectoparasites such as ticks, fleas, and mosquitoes [1]. Control of these infectious agents is important because some are responsible for serious zoonotic diseases (e.g., *Anaplasma phagocytophilum* and *Borrelia burgdorferi*). However, their control can be a highly complex process since they show a wide geographical distribution and the clinical signs in infected dogs may vary significantly [2,3].

CVBD may show nonspecific clinical signs or clinical-pathological abnormalities, which makes the diagnosis of a CVBD extremely complex. In addition, animals may even present a varied clinical picture [4,5].

Canine babesiosis is a common and clinically significant tick-borne disease caused by hematozoan parasites of the genus *Babesia* [6]. Two morphologically distinct forms of the erythrocytic stage in the canine host have been recognized in early studies that led to the naming of the larger form, measuring approximately 3–5 µm as *Babesia canis* and the smaller (1–3 µm) as *B. gibsoni*. On the basis of cross-immunity, serological testing, vector specificity and molecular phylogeny *B. canis* was reclassified into three sub-species: *B. canis canis*, *B. canis rossi*, and *B. canis vogeli*. All of them are now considered to be separate species [7,8]. Even though the three species have been detected in Europe, only *B. canis* has been found in dogs in Poland [9,10]. These parasites are also the most common etiologic factor of babesiosis in dogs in other parts of Europe [11–13]. Clinically, these pathogens cause remittent fever, progressive anemia, haemoglobinuria, and marked splenomegaly and hepatomegaly in dogs, and in some cases the death of infected animals [14,15].

Infections with *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, have been increasingly diagnosed in both pet and livestock animals [16,17]. This pathogen has also been found in the blood of game animals [18,19]. *Anaplasma phagocytophilum* usually causes an acute infection in dogs, characterized by fever and thrombocytopenia. This pathogen was recently observed in 10.3% of *Ixodes ricinus* ticks studied in the eastern part of Poland [20].

Lyme disease is an infectious disease caused by the spirochetes *Borrelia burgdorferi sensu lato* complex, transmitted by ticks of the genus *Ixodes* [21]. *Borrelia burgdorferi* affects a wide range of hosts, mainly humans and dogs. In dogs, Lyme disease can produce chronic weakness with nonspecific clinical signs (fever, muscle, joint pain). Although some dogs show clinical signs, mostly the infection is subclinical [21].

Standard diagnosis of CVBD is the serological examination of the animals (Lyme disease) or identification of pathogens in Giemsa-stained thin-film blood smears examined by microscopy (*Babesia*, *Anaplasma*). However, the detection of parasites and rickettsiae using the latter technique is difficult in dogs with unapparent or chronic infections since the level of parasitemia and bacteriemia is very low [22]. Molecular diagnosis from blood samples using PCR technique and its varieties (real-time PCR, LAMP etc.) is characterised by relatively high sensitivity in identifying infections. These methods may also present false-negative results, if the pathogens are accumulated in the spleen, for instance [6,23]. Therefore, the development of a highly specific and sensitive system for the diagnosis of CVBD is required.

Mass spectrometry (MS) – based proteomics analyses offer new approaches to identify biomarkers for the detection of disease and for monitoring therapeutic and toxic outcomes. MALDI-TOF (Matrix Assisted Laser Desorption Ionization with Time of Flight detector) – based proteome profiling of serum, other bio-fluids as well as tissue sections have been widely employed for pattern-based diagnostics and biomarker discovery. MALDI spectral features correspond to a subset of proteins present in the sample and collectively constitute proteomic patterns that represent different biological states [24–26].

The aim of the study was to apply the MALDI-TOF technique to demonstrate changes in the serum protein profile of dogs infected with *A. phagocytophilum*, *B. canis* or *B. burgdorferi.*

Materials and Methods

Animals used in the study. The study was performed in the Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, and included 70 dogs of various breed and sex, divided into 4 groups. Group 1, the control group (n=10, five females and five males, aged 6 months–7 years), consisted of healthy dogs (the dogs came to the Clinic for vaccination. Their health status was assessed based on the results of clinical and haematological examinations). Group 2, the study group (n=20, seven females and thirteen males, aged 1–10 years), consisted of dogs naturally infected with *B. canis*. Group 3 (n=20, eleven females and nine males, aged 4 months–6 years) included dogs naturally infected with *A. phagocytophilum*. Group 4 (n=20, six females and fourteen males, aged 1–8 years) included dogs naturally infected with *B. burgdorferi*. All dogs from groups 2, 3 and 4 showed symptoms of babesiosis, anaplasmosis and borreliosis, and were tested positive by both thin blood smears and specific PCR for *B. canis* and *A. phagocytophilum* and using both ELISA and Western blot for *B. burgdorferi*.

PCR for *B. canis* and *A. phagocytophilum* were performed as previously described by Adaszek et al. [10], and Dzięgiel et al. [27], and ELISA and Western blot tests for *B. burgdorferi* were performed as previously described by Adaszek et al. [28].

Blood samples were collected from animals from each group into test tubes containing a coagulation accelerator. Samples were then centrifuged to obtain serum that was used for proteomic testing.

MALDI-TOF MS. 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 sera were mixed with the SA matrix (sinapinic acid) suspended in a TA 30 solution 30 (70:30 0.1% TFA in H_2O:ACN). A layer of the SA matrix suspended in EtOH HPLC Grade

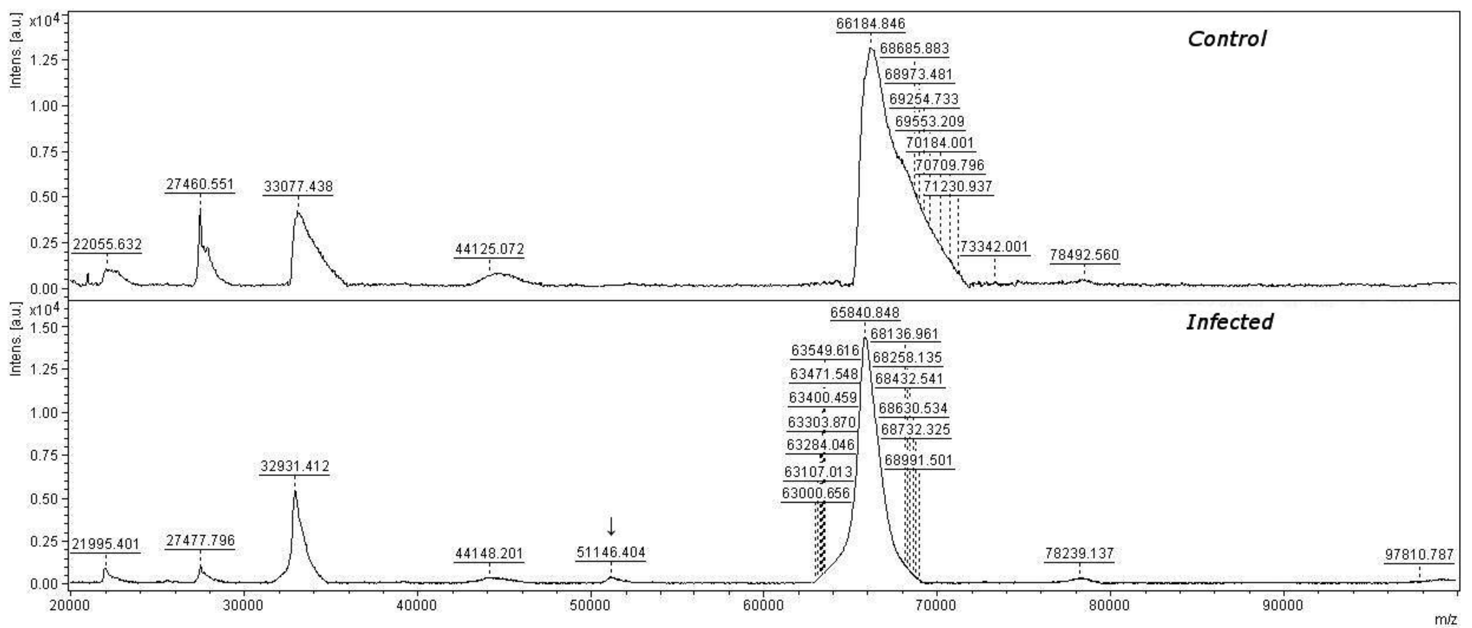

Fig. 1. MALDI-TOF mass spectrometry analysis of blood serum of dogs infected by *Babesia canis canis* and healthy animals. Spectra were acquired in linear positive mode in a mass range of 20–100 kDa. An additional protein fraction (51–52 kDa – arrow) was detected in serum of dogs with babesiosis.

(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 Ultraflextreme 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.

Statistical analysis. The Kappa value for the agreement between MALDI-TOF and PCR was calculated as previously described [29].

Results

Proteomic analysis demonstrated the presence of eight protein fractions ranging from 20 to 100 kDa in serum samples obtained from all the tested animals, both in the three study groups and in the control group. An additional protein fraction of approximately 51–52 kDa (Fig. 1) was only found in serum samples from dogs with babesiosis. This protein was not detected in the serum of any of the control group dogs, as well as in the serum of dogs infected with *A. phagocytophilum* or *B. burgdorferi*. This indicates that the fraction discussed may be used to identify dogs infected with *B. canis* protozoa. All animals from group 1, 3 and 4 had the same serum profile.

The Kappa value for agreement between MALDI-TOF and PCR using positive and control samples was perfect (κ=1.000).

Discussion

MALDI-TOF mass spectrometry technique with regard to CVBD is more often used to identify the species of ticks, and to demonstrate the presence of certain pathogens in these vectors [30–32], as well as to study the proteome profile of pathogens transmitted by ticks [33,34].

However, information concerning the use of mass spectrometry in testing serum protein profiles in dogs infected with *B. canis* is still very limited. Kuleš et al. [35] used MALDI-TOF to monitor the clinical course and pathogenesis of canine babesiosis. Own observations indicate that in reference to canine babesiosis, mass spectrometry is a sensitive diagnostic technique. Results of our study were 100% compliant with molecular (PCR) testing.

The protein fraction of a similar weight – 51–52 kDa, observed in our study in sera from twenty *B. canis*-infected dogs, was also found in soluble parasite antigens (SPA) obtained from the supernatant of the protozoa *in vitro* culture [36]. This fraction appeared to be strongly immunogenic,

which was confirmed by Western blot tests. It was the proteins of 51–52 kDa that caused the strongest reaction with serum samples obtained from the dogs vaccinated with SPA [36,37]. Similar results in group of 15 dogs were previously obtained by Adaszek et al. [38]. 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 disease marker.

The proteomic analysis of sera from dogs infected with *A. phagocytophilum* or *B. burgdorferi* did not reveal any markers associated with these diseases. In the present study, no differences that could give rise to specify a signal differentiating healthy individuals with infected with rickettsial and spirochetes were observed. It is recommended to conduct further analysis according to the adopted top-down analytical strategy.

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