

**FIRST REPORT ABOUT CANINE INFECTIONS WITH *CANDIDATUS MYCOPLASMA HAEMATOPARVUM* IN SERBIA**

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(Received 2<sup>nd</sup> May 2010)

*There are two species of haemotropic mycoplasmas that infect dogs which are known so far. These are *Mycoplasma haemocanis* and "*Candidatus Mycoplasma haematoparvum*". In the case of dogs, the clinical picture of these infections manifests itself in immunocompromised dogs, although the organisms are also present in immunocompetent dogs which do not show any signs of infection. The vector for these microorganisms is the tick *Rhipicephalus sanguineus*. The goal of this research was to confirm the presence of canine infections with haemotropic mycoplasmas in our geographic area, regarding the facts that the vector of canine haemotropic mycoplasmas, the *Rhipicephalus sanguineus* is widely spread in nature, and that the epidemic indications and the clinical signs of canine infections are also present. By means of light microscopic examination of stained peripheral canine blood smears, scanning electron microscopic examination of canine erythrocytes, as well as PCR and sequence analysis of the genome, the presence of canine infections with "*Candidatus Mycoplasma haematoparvum*" in our geographic area has been identified for the first time.*

*Key words: anaemia, "*Candidatus Mycoplasma haematoparvum*", dogs, *Haemobartonella*, haemotropic mycoplasmas, *Mycoplasma haemocanis**

INTRODUCTION

Haemotropic mycoplasmas, or haemoplasmas, are wall-less bacteria localized on the erythrocyte surface of mammals and they are uncultivable. The organisms can be observed in peripheral blood smears by light microscopy. They are visible as rods, cocci and ring forms (Messick, 2004). Until recently, there was only one species known, *Mycoplasma haemocanis* (formerly *Haemobartonella canis*), which infects dogs all over the world. This organism was first observed by Kikuth in 1928. It is a relatively large haemoplasma (0.3-2.0 µm in diameter), which

characteristically forms long chains on the surface of dogs erythrocytes. Subclinical infection can occur in immunocompetent dogs. In the case of immunocompromised dogs, this infection clinically manifests itself in signs of anaemia, loss of weight, fever, anorexia and lethargy (Messick *et al.*, 2002). Recently, Sykes *et al.* (2004) have identified a novel haemotropic mycoplasma in a splenectomized dog with a neoplasm. The vector of canine haemoplasmas is the tick *Rhipicephalus sanguineus*, whose main geographical distribution is connected with the Mediterranean and sub-Mediterranean climates. Regarding the fact that haemoplasmas can not cultivate in *in vitro* conditions, most of the studies were based on the cytological identification of the organisms in canine peripheral blood smears. This method of direct diagnosis is of low specificity and sensitivity. The method of choice in the diagnosis and analysis of this infection is PCR (Wengi *et al.*, 2008). PCR is a highly sensitive and specific diagnostic method, which can also be applied to the diagnosis of latent dog infections, when the clinical signs of infection with haemoplasmas do not manifest themselves (Brinson and Messick, 2001). The goal of this research is to diagnose the presence of canine infections with haemotropic mycoplasmas in our geographic area, regarding the fact that the vector of canine haemotropic mycoplasmas, *Rhipicephalus sanguineus* is widely spread in nature, and the fact that the epidemic indications and clinical signs of dog infections are also present.

#### MATERIALS AND METHODS

##### *Samples*

For this research, blood samples of 12 American Stafford Terrier breed dogs were used, obtained by venipuncture of the *v. radialis*. Samples of 3 mL whole vein blood were collected into vacutainers containing EDTA, and samples of 3 mL into vacutainers containing Lithium Heparin. The native smears of peripheral blood of the same dogs were prepared immediately after venipuncture.

##### *Light Microscopy of Peripheral Blood Smears*

After drying, the peripheral blood smears were fixed for 5 minutes in methanol and stained for 10 minutes according to Giemsa's method. After rinsing and drying, the peripheral blood smears of dogs were examined by immersion microscopy using a magnification of 1000x.

##### *Scanning Electron Microscopy of Erythrocytes*

After sedimentation of erythrocytes in vacutainers containing Lithium Heparin, the erythrocytes were prepared for SEM examination by double rinsing with physiological saline solution for 5 minutes. Afterwards, they were fixed for two hours in 2.5% glutaraldehyde solution in distilled water at 4°C. After fixation had been completed, the erythrocyte suspensions were rinsed in distilled water three more times for 30 minutes. As rinsing had been finished, the erythrocytes were dehydrated for 20 minutes in ethanol solutions of the following concentrations: 25%, 50%, 75%, 95% and 100%. After that, the erythrocyte suspensions were placed on the SEM stage. Coating in pure gold was performed for 180 seconds at

30 milliamperes and WD 50 mm (SCD005, BAL-TEC). The microscope model used for SEM was JSN – 6460 LV (JOEL).

#### *DNA Extraction and PCR Amplification*

200 µL EDTA whole blood were applied for DNA isolation and purification using the QIAamp® DNA Blood Mini Kit according to the manufacturer's instructions (Vet Med Lab GmbH). The PCR assay for DNA detection and differentiation of *Mycoplasma haemocanis* and "*Candidatus Mycoplasma haematoparvum*" was modified according to the previously described method (Jensen *et al.*, 2001). In order to increase the analytical specificity of the PCR assay, Restriction Fragment Length Polymorphism (RFLP) analysis was performed on the positive PCR amplicons. The PCR amplification products were identified by ethidium bromide fluorescence after electrophoresis in 2% agarose gels. For the detection of the RFLP products, 2% metaphor agarose gels were used. A known amount of plasmid DNA, near the detection limit of the PCR (25 copies), was included in each PCR as the positive and sensitive control. A blank control (no template control, NTC), as well as pure water treated as a patient sample were included as negative and extraction controls. All DNA preparations were checked for the presence of inhibitory substances prior to PCR analysis by measuring the spiked extraction controls according to the Quality Standards for Microbiological Diagnostics of Infectious Diseases (MIQ). Samples with inhibitory substances were excluded.

#### *Genome Sequence Analysis*

The BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Germany) was applied, according to the manufacturer's instructions, to the sequences of both DNA strands of the PCR amplicon. When compared with the DNA sequence databases of GenBank, EMBL, DDBJ, and PDB by BLASTN - BLASTN 2.2.16 (Altschul *et al.*, 1997) the analysed 150 bp revealed a homology of 100% to '*Candidatus Mycoplasma haematoparvum*' (GenBank Acc. No AY383241.1).

## RESULTS

The light microscopic examination of peripheral blood smears stained according to Giemsa showed the presence of basophilic coccoid forms on the erythrocyte surface of six dogs. During the same examination, the presence of echinocytes and spherocytes was revealed in every dog which had haemoplasmas on the erythrocytes (Figure 1).

The method of scanning electron microscopy revealed the presence of haemoplasmas of 290 nm and 269 nm in diameter on the erythrocyte surface, as well as the presence of echinocytes and spherocytes (Figure 2).

The presence of DNA *Mycoplasma haemocanis* in the blood of dogs which contained haemoplasmas (revealed by microscopic examination) was proved by PCR analysis.

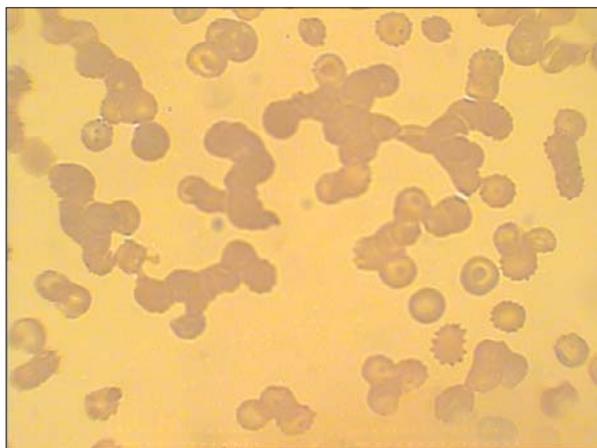


Figure 1. Blood smear of infected dog stained with Giemsa demonstrating presence of haemotropic mycoplasmas on erythrocytes and echynocytes with haemotropic mycoplasmas. Magnification 1000X



Figure 2. Scanning Electron Microscopy image showing presence of haemotropic mycoplasmas on the surface of one erythrocyte. Magnification 40.000X

The partial sequence analysis of the 16S rRNA gene (both sequences had 150 bp) indicated a homology of 100% between the PCR amplicons of the positive samples and "*Candidatus Mycoplasma haematoparvum*".

## DISCUSSION

This is the first case of canine infection with "*Candidatus Mycoplasma haematoparvum*" in our geographic area. This organism, named "*Candidatus Mycoplasma haematoparvum*" is smaller (0.3 µm in diameter) and does not form chains on the erythrocyte surface of dogs (Sykes *et al.*, 2005). The infection has been confirmed by methods of molecular biology (Jensen *et al.*, 2001). In France, a 15.4% prevalence of haemoplasma infection in dogs, as well as the presence of coinfection of both haemoplasmas, have been registered using the PCR test (Kenny *et al.*, 2004). Further researches on haemotropic mycoplasmas are indispensable, regarding that: haemoplasmas might act as cofactors in the progression of retroviral, neoplastic and immunologically mediated diseases; the factors of the virulence and pathogenic mechanisms in the development of these infections, as well as the functions of the immunologic system, which is in this case again responsible for the occurrence of new opportunistic infections, are not known (Messick, 2004). Until now, there is no evidence that canine haemoplasmas cause human diseases, but regarding the fact that feline and swine haemotropic mycoplasmas have zoonotic potential, future researches should be conducted in this direction (Wu *et al.*, 2006; dos Santos *et al.*, 2008).

## ACKNOWLEDGEMENTS:

This study was supported by a grant from the Ministry of Science and Technological Development Republic of Serbia, number 20124, year 2008.-2011.

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#### PRVI PODACI O INFEKCIJI PASA SA *CANDIDATUS MYCOPLASMA HAEMATOPARVUM* U SRBIJI

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#### SADRŽAJ

Poznate su dve vrste hemotropnih mikoplazmi koje inficiraju pse. To su *Mycoplasma haemocanis* i "*Candidatus Mycoplasma haematoparvum*". Klinička slika ovih infekcija, kod pasa, se ispoljava kod imunokompromitovanih jedinki, iako su uzročnici prisutni i kod imunokompetentnih pasa bez ispoljenih znakova infekcije. Vektor ovih uzročnika je krpelj *Rhipicephalus sanguineus*. Kao cilj istraživanja, postavljeno je utvrđivanje prisustva infekcije pasa sa hemotropnim mikoplazmama na našem geografskom području, s obzirom na činjenicu da je vektor hemotropnih mikoplazmi pasa *Rhipicephalus sanguineus* široko rasprostranjen u prirodi, kao i da postoje epidemiološke indikacije i klinički znaci infekcije pasa. Primenom svetlosne mikroskopije obojenih razmaza periferne krvi pasa, skenirajućom elektronskom mikroskopijom eritrocita pasa, kao i primenom PCR i sekvencionirane analize genoma, utvrđeno je, po prvi put, prisustvo infekcije pasa sa "*Candidatus Mycoplasma haematoparvum*" na našem geografskom području sa prevalencijom infekcije od 50 % u populaciji ispitivanih pasa.