UDC 619:616-07:597.887.111:636.7(497.11)

CLINICAL INVESTIGATION AND THERAPY OF CANINE INFECTION WITH *CANDIDATUS MYCOPLASMA HAEMATOPARVUM* IN SERBIA

Potkonjak A.[1], Lako B.[1], Belic R.[2], Toplaski B.[2], Stevancevic O.[1], Gerilovych A.[3]

[1] Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Novi Sad, Serbia; e-mail: ale@polj.ns.ac.yu

[2] Veterinary Clinic NS-VET, Novi Sad, Serbia; e-mail: nsvet@neobee.net

[3] National Scientific Center "Institute of Experimental and Clinical Veterinary medicine", Kharkov, Ukraine; e-mail: antger@vet.kharkov.ua

Haemotropic mycoplasmas (haemoplasmas) are small bacteria that reside on the surface of red blood cells and mediate haemolytic anemia in a wide range of vertebrate hosts. Until recently it was believed that only Mycoplasma haemocanis, formerly referred as Haemobartonella canis, could infect dogs. However, a new canine haemoplasma, named Candidatus Mycoplasma haematoparvum, has been described. In this study, several dogs from one breeding facility in Serbia that showed clinical signs of haemoplasma infection were tested for the presence of this infectious agent. The presence of Candidatus Mycoplasma haematoparvum in these animals was confirmed by blood exam (Giemsa stained blood smears, Scanning Electron Microscopy) as well as using molecular biology techniques (PCR, nucleic acid sequencing). Treatment of infected dogs with doxycycline (10 mg/kg of body weight) for 21 days led to remission of infection. Thus, we reported for the first time infection of dogs with Candidatus Mycoplasma haematoparvum in southeast part of Europe.

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 [5]. 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 erythrocyte surface of dogs. 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 [6]. Recently, Sykes and his colleagues have identified a novel haemotropic mycoplasma in a splenectomized dog with a neoplasm [7]. 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 [9]. 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 the infection with haemoplasmas do not manifest themselves [2]. 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, the *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. *Clinical examination.* Standard clinical examination of 12 American Stafford Terrier bread dogs was made. Function of all organ systems and basic physiological functions were checked including, body temperature, respiration frequency, heart beating etc. Abdominal ultrasound imaging was also performed on all dogs.

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 the 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 1000×.

Scanning electron microscopy of erythrocytes. After the sedimentation of the erythrocytes in the 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 the temperature of 4 °C. After the fixation had been completed, the erythrocyte suspensions were rinsed in distilled water three more times for 30 minutes. As the 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 ma 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 [3]. 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 positive and sensitive controls. 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 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 v 1.1 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 [1] the analyzed 150 bp revealed a homology of 100% to `*Candidatus Mycoplasma haematoparvum*` (GenBank Acc. No AY383241.1).

Results and discussion. American Stafford Terrier bread female dog, 4 years old, was examined on request of his owner. The dog was lethargic for previous several days showing no interest for food, apathy and reduced physical condition. The examined dog was imported in Serbia about one month ago and upon arrival its health status was thoroughly checked. On its arrival health check, the dog showed no sign of any existing illness, the blood count and biochemistry tests were physiologically normal and body weight was 24 kg. Afterwards, the dog was vaccinated and treated against infective diseases and parasites. According to his owner words, the dog wasn't bitten by any tick in previous period, although it spend some time on open fields and meadows on which presence of ticks and other arthropods is to be expected. The owner also reported that almost one year ago another diseased dog of same bread with similar symptoms died in the same breading facility. Since its last health examination the dog showed loss of body weight for 6 kg. The dog was also febrile with body temperature 38.6° C, with intensive respiration and pale buccal mucosa and pale conjunctivae. Detailed analysis of skin and hair didn't show presence or bite of ectoparasites or obvious external bleeding. Hepatosplenomegaly was identified by palpation and abdominal sonography. Other organs or organ systems were not affected. Repeated examination on next day showed same symptoms with even higher body temperature of

39.5 °C. Additional blood check demonstrated leucocytosis (WBC 16,2Ч10^9/l), anemia (RBC 2.11×10^{12}/l), thrombocytopenia (31×10^9/l), hemoglobin concentration of 58 g/l, while the rest of hematological and biochemical parameters were physiologically normal. Another female dog (5 years old) from the same breeding facility with similar symptoms was reported in the same time. The dog was lethargic and anorexic, febrile (body temperature 40.0 °C) with signs of hepatomegaly. The dog gave birth on previous day and out of 14 delivered litters, 12 died right after birth. Blood was taken from diseased dog and blood count and biochemical analysis were made. It was found that the dog has leukocytosis (WBC 11.4×10^9/l), anemia (RBC 3.58×10^{12}/l), thrombocytopenia (130×10^9/l) and bilirubinemia (14.1 μmol/l), while the rest of the parameters showed normal physiological values. Because the rest of dogs from the same animal facility were in contact with diseased animals, they were carefully examined for the presence of same symptoms. Out of 10 dogs from animal facility, four dogs showed mild anemia in blood check test, while other six were healthy. There was no tick bite reported on these dogs in last one month, although they were spending some time in open areas in which presence of ticks is to be expected. To detect potential blood parasites, blood smears from all affected animals were prepared, stained by Giemsa and analyzed by light microscopy. We detected basophilic cocoon shape bodies on erythrocytes of all diseased dogs. In blood of one of diseased animals we noticed the presence of acantocytes. To further study morphology of detected parasites, we performed Scanning Electron Microscopy. Using this techniques we distinguished "haemoplasmas" with diameter of 290 nm attached to the surface of red blood cells plasma membrane. Detected bacteria were present in doublets or singly and on some of slides we were able to notice presence of acantocytes and spherocytes. Higher specificity and sensitivity of PCR technique, in comparison with microscopy, allowed us to more precisely evaluate the infection agent. DNA was isolated from the blood samples of affected dogs and subjected to PCR assay followed by the sequencing of 150 bp long amplicon. Tests were performed by Vet Med Labor GmbH, Germany. The obtained amplicon sequence was subjected to phylogenetic analysis with related canine and feline haematoplasma sequences derived from NCBI BLAST searches. Performed phylogenetic analysis of sequences showed 100 % homology with *Candidatus Mycoplasma haematoparvum*, 99 % homology with *Candidatus Mycoplasma haemominutum,* 95 % homology with *Candidatus Mycoplasma kahanei*, 93 % homology with *Mycoplasma suis*, 92 % homology with *Mycoplasma ovis* etc. Thus, obtained results strongly suggest that dogs were infected by "*Candidatus Mycoplasma haematoparvum*" species. Upon applied therapy with doxycycline administration for 21 days (10 mg/kg of body weight) all infected dogs showed remission of disease. Upon applied therapy with doxycycline administration for 21 days (10 mg/kg of body weight) all infected dogs showed remission of disease. Until recently, only one canine haemoplasma had been described and was later renamed to *Mycoplasma haemocanis* [6]. "*Candidatus Mycoplasma haemoparvum*" also known as "*Cadidatus Mycoplasma Haematoparvum*" is relatively novel described haemoplasma [8]. The distribution of this microorganism in population of dogs was confirmed up to date in France and Switzerland [4, 9]. It is also suggested that distribution of this agent is related to climate zones that coincides with presence of *Rhipicephalus sanguines* [9]. This is first report on "*Candidatus Mycoplasma haematoparvum*" infection in South East Europe. Using clinical examination and laboratory testing, we confirmed infection of several dogs with this agent. It is hard to claim that the infectious agent was introduced into the breeding facility by imported dog since dog had no signs of disease at the time of import. Still, since less than one month passed prior the appearance of the first symptoms, this possibility cannot be excluded. The mean of "*Candidatus Mycoplasma haematoparvum*" transmission among dogs in the breeding facility also remains to be clarified. Out off 12 analyzed dogs, 6 dogs were infected, which is determined using routine microscopy technique and PCR assay. Two of infected dogs developed classical clinical symptoms of disease. While PCR assay demonstrated that infective agent belong to canine haemoplasmas, partial sequence analysis of 16S rRNA gene specifically pointed to presence of "*Candidatus Mycoplasma haematoparvum*". We were able to detect small coccus-shaped organisms of approximately

0.3 μm in size attached to the surface of erythrocytes. The size of bacteria and their appearance in doublets or singly is in accordance with identification of these haemoplasmas as "*Candidatus Mycoplasma haematopravum*" species. This is the first report on "*Candidatus Mycoplasma haematopravum*" in Southeast Europe. The implication of this finding may overcome veterinary medicine interest as it is known that some of the Mycoplasms have a significant zoonotic potential. Having in mind an assumption that haemoplasmas might contribute to the progression of retroviral, neoplastic and autoimmune diseases. A huge gap in our knowledge on pathogenic mechanisms of disease and role of immune systems in infection protection clearly suggest a need for a further study on this topic.

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

References

1. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs [Text] / Altschul S.F. [et al.] // Nucleic Acids Res. — 1997. — Vol. 25. — P. 3389–3402. ***2.*** Brinson, J.J. Use of a polymerase chain reaction assay for detection of Haemobartonella canis in a dog [Text] / Brinson J.J., Messick J.B. // J. Am. Vet. Med. Assoc. — 2001. — Vol. 218. — P. 1943–1945, 1936. ***3.*** Jensen, W.A. Use of a polymerase chain reaction assay to detect and differentiate two strains of Haemobartonella felis in naturally infected cats [Text] / Jensen W.A., Lappin M.R., Kamkar S., Reagan W.J. // Am. J. Vet. Res. — 2001. — Vol. 62. — P. 604–608. ***4.*** Kenny, M.J. Demonstration of two distinct hemotropic mycoplasmas in French dogs [Text] / Kenny M.J., Shaw S.E., Beugnet F., Tasker S. // J. Clin. Microbiol. — 2004. — Vol. 42. — P. 5397–5399. ***5.*** Messick, J.B. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential [Text] / Messick J.B. // Vet. Clin. Pathol. — 2004. — Vol. 33. — P. 2–13. ***6.*** 'Candidatus mycoplasma haemodidelphidis' sp. nov., 'Candidatus mycoplasma haemolamae' sp. nov. and Mycoplasma haemocanis comb. nov., haemotrophic parasites from a naturally infected opossum (Didelphis virginiana), alpaca (Lama pacos) and dog (Canis familiaris): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas [Text] / Messick J.B. [et al.] // Int. J. Syst. Evol. Microbiol. — 2002. — Vol. 52. — P. 693–698. ***7.*** Identification of a novel hemotropic mycoplasma in a splenectomized dog with hemic neoplasia [Text] / Sykes J.E. [et al.] // J. Am. Vet. Med. Assoc. — 2004. — Vol. 224. — P. 1946-1951, 1930-1931. ***8.*** Sykes, J.E. 'Candidatus Mycoplasma haematoparvum', a novel small haemotropic mycoplasma from a dog [Text] / Sykes J.E., Ball L.M., Bailiff N.L., Fry M.M. // Int. J. Syst. Evol. Microbiol. — 2005. — Vol. 55. — P. 27–30. ***9.*** Real-time PCR-based prevalence study, infection follow-up and molecular characterization of canine hemotropic mycoplasmas [Text] / Wengi N. [et al.] // Vet. Microbiol. — 2008. — Vol. 126. — P. 132–141.

КЛИНИЧЕСКОЕ ИССЛЕДОВАНИЕ И ЛЕЧЕНИЕ ИНФЕКЦИИ CANDIDATUS MYCOPLASMA HAEMATOPARVUM У СОБАК В СЕРБИИ

Потконьяк А.[1], Лако Б.[1], Белич Р.[2], Толпаски Б.[2], Стеванчевич О.[1], Герилович А.[3]

[1] Кафедра ветеринарной медицины, Факультет сельского хозяйства, Университет Нови Сад, Сербия. [2] Ветеринарная клиника NS-VET, Нови Сад, Сербия. [3] Национальный научный центр «Институт экспериментальной и клинической ветеринарной медицины», Харьков, Украина

Гемотропические микоплазмы (гемоплазмы) – это маленькие бактерии, которые обитают на поверхности эритроцитов и вызывают гемолитическую анемию в широком ряде организмов позвоночных. До последнего времени предполагалось, что только Mycoplasma haemocanis, которая относится к Haemobartonella canis, может заражать собак. Однако была охарактеризована новая микоплазма собак, названная Candidatus Mycoplasma haematoparvum. Исследование проводилось на нескольких собаках одного хозяйства Сербии. По клиническим признакам было изучено наличие возбудителя инфекции. Присутствие Candidatus Mycoplasma haematoparvum у этих животных было подтверждено анализом крови (окрашивание мазков крови красителем Гимза, сканирующая электронная микроскопия), а также использованием технологий молекулярной биологии (ПЦР, секвенирование нуклеиновой кислоты). Ремиссии инфекции предшествовало лечение инфицированных собак доксициклином (10 мг/кг на вес тела) в течение 21 суток. Таким образом, мы впервые установили инфицирование собак Candidatus Mycoplasma haematoparvum в юго-восточной части Европы.