Serum levels of the chemokines keratinocyte chemoattractant and interleukin-8 in dogs naturally infected with *Babesia canis canis*

Iva Mayer1*, Krešo Bendelja2, Mirna Brkljačić1, Martina Crnogaj1, Iva Šmit1, Marin Torti1, Velimir Sušić4, Renata Barić Rafaj3, and Vladimir Mrlijak1

1Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia
2Institute of Immunology, Zagreb, Croatia
3Department of Chemistry and Biochemistry, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia
4Department of Animal Husbandry, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

ABSTRACT

Canine babesiosis, caused by *Babesia canis canis*, is one of the commonest canine diseases in Croatia. It is known that systemic inflammatory response syndrome (SIRS), which is a hallmark of babesiosis, is regulated by the host production of several pro-inflammatory and anti-inflammatory cytokines that have been implicated as playing a critical role in the pathogenesis of the disease. In the course of larger research, serum concentrations of CXC chemokines keratinocyte chemoattractant (KC) and interleukin-8 (IL-8) were determined. The aim of the present study was to determine serum chemokine concentrations and to compare values between healthy and dogs with babesiosis. An additional aim was to determine if KC and IL-8 serum concentrations are an important contributing factor in the development of complicated babesiosis, and whether they affect the outcome of the disease. The results suggest that a higher serum concentration of KC-like is connected with the severity of anemia, leukocytosis and development of a complicated form of babesiosis. The IL-8 concentration positively correlates with granulocyte-lymphocyte ratio. In addition concentration of both chemokines was higher in dogs with complicated babesiosis. Therefore, the concentration of the observed chemokines could be considered as an important factor in the pathogenesis of canine babesiosis.

Key words: keratinocyte chemoattractant, interleukin-8, inflammatory response, dog, babesiosis

*Corresponding author:
Iva Mayer, DVM, PhD, Assistant, Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone: +385 1 23 90 357; E-mail: imayer@vef.hr
Introduction

Babesiosis, caused by the haemoprotozoa Babesia canis canis (B. canis canis), has been reported to be a significant cause of canine morbidity and mortality in Croatia (CACCIO et al., 2002; MATIJATKO et al., 2009; BRKLJAČIĆ et al., 2010). Clinical manifestation of canine babesiosis can vary from very mild to very severe, and can result in various outcomes. It is known that immunity to the protozoan parasite Babesia is mediated by both innate and adaptive immune mechanisms (AHMED, 2002). In terms of clinical signs, canine babesiosis can be uncomplicated or complicated (JACOBSON, 2006). Although primarily considered as a predominantly hemolytic disease, recent studies have confirmed that the pathogenesis of complicated canine babesiosis is largely determined by the generalized and uncontrolled inflammatory response of the host (JACOBSON and CLARK, 1994; BOOZER and MACINTIRE, 2003; MATIJATKO, 2003). In fact, the acute phase response (APR) which results in systemic inflammatory response syndrome (SIRS), is considered to be a hallmark of canine babesiosis. On this basis, canine babesiosis is defined as protozoal sepsis and its pathogenesis is similar to other septic conditions (JACOBSON et al., 2006; MATIJATKO et al., 2007).

As a response to the presence of parasites in the circulation, the host produces a variety of pro-inflammatory and anti-inflammatory factors, in order to recognize and control the invasion, as well as to repair affected tissue. Cytokine production plays a critical role in the host defense and the development of SIRS in babesiosis (KRAUSE et al., 2007). A disturbed balance between pro-inflammatory and anti-inflammatory cytokines results in the uncontrolled progression of the inflammatory response and subsequent multiple organ dysfunction syndrome (MODS) (ABBAS et al., 2000).

The inflammatory response of the host is determined by the complex interaction between numerous groups of cytokines. Chemokines are a large subfamily of cytokines, with chemotactic activity for different cell types, including: neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts, and keratinocytes (LOCATI et al., 2005; SCOTT et al., 2005). Apart from chemotaxis, they also perform a variety of functions, including regulation of hematopoiesis, fibrosis, and angiogenesis (HE et al., 2007). In terms of their function, chemokines may be divided into two main groups: homeostatic and pro-inflammatory chemokines. In general, homeostatic chemokines are constitutively expressed in specific tissues or organs, whereas production of inflammatory chemokines by many cell types is induced during the inflammatory response.

Interleukin-8 (IL-8) and keratinocyte chemoattractant (KC) are considered typical, but not exclusively, pro-inflammatory chemokines. Their main function is chemotraction and recruitment of neutrophils during immune responses (LOCATI et al., 2005). Increased levels of IL-8 are reported in human medicine in patients with malaria, acute pancreatitis, severe sepsis and MODS, and are considered to be a predictor of a bad outcome.
I. Mayer et al.: Serum levels of chemokines KC and IL-8 in dogs naturally infected with *Babesia canis canis*

(FRIEDLAND, 1993; RAU et al., 1997; HAMANO et al., 1998; BOZZA et al., 2007). Likewise, studies of anaplasmosis have reported elevated serum activity of IL-8 in humans and horses infected with *Anaplasma phagocytophilum* (KIM et al., 2001; AKKOYUNLU et al., 2001). Recent studies have confirmed that plasma IL-8 concentrations are increased in dogs with spirocercosis (DVIR et al., 2012).

Expression of KC is induced by the pro-inflammatory activity of monocytes and endothelium. Its primary function is chemotaxis and activation of inflammatory cells (FRINK et al., 2007). To the author’s knowledge, there have been very few investigations about the role of KC in dogs with a naturally occurring disease. In human medicine a higher concentration of KC in the BAL during acute lung injury has been reported (LEE et al., 2009). On the basis of murine and human studies, KC is also considered to be an attractive early biomarker of acute kidney injury (MOLLS et al., 2006). The study performed by FRINK et al. (2007) showed that KC plays a pivotal role in neutrophil infiltration and organ damage after trauma-hemorrhage and resuscitation.

The aim of this study was to determine and compare IL-8 and KC serum concentrations in healthy dogs and dogs naturally infected with *Babesia canis canis*. Also, we wanted to investigate whether serum IL-8 and KC concentrations are correlated with changes in hematology and biochemistry parameters and whether they contribute to the development of complicated babesiosis.

**Materials and methods**

**Animals.** Forty-seven dogs were retrospectively included in this study and divided into two main groups. Group 1 included 31 dogs naturally infected with *B. canis canis*. Group 2 included 16 healthy dogs that were used as healthy controls. In the control group, the dogs were deemed healthy based on their history, physical and laboratory findings (complete blood count, routine serum biochemistry and urinalysis). All of the obtained data were within the reference ranges. The study protocol was approved by the Ethics Committee for Animal Experimentation, Faculty of Veterinary Medicine, University of Zagreb, Croatia. Healthy dogs were clinically examined and sampled before prophylactic single application of imidocarb dipropionate (Imizol®, Schering-Plough), as well as 1 and 6 days after application. Imidocarb dipropionate was administered for prophylactic purposes at the owners’ request subcutaneously (s.c.) in a dose of 6.6 mg/kg.

**Diagnosis of babesiosis.** Babesiosis was diagnosed by demonstrating *B. canis* in Romanowsky stained blood smears. Afterwards babesia were characterized by the polymerase chain reaction method (PCR). Furthermore, on the basis of clinical, hematological and serum biochemistry findings, dogs with babesiosis were divided into a group with a complicated form of the disease and a group with an uncomplicated form. A single dose (6.6 mg/kg subcutaneously) of Imizol® was administered to all the dogs on
the day of admission, after establishing the diagnosis of babesiosis. Additional treatment consisted of various fluids (colloid and crystalloid therapy), and whole blood transfusion when it was indicated. Whole blood and serum samples were taken from the cephalic vein at admission, and 1 day and 6 days after admission.

**Haematological analysis.** Samples taken from dogs with babesiosis at admission were used for routine hematology and biochemistry profiles, as well as for establishing the diagnosis. Blood smears and PCR were preformed from hematology samples taken at admission. Whole blood samples were placed in tubes with ethylenediaminetetraacetic acid (EDTA) for hematological analysis. White blood cell count (WBC), platelet count and hematocrit (HCT) were determined using a Horiba ABX automatic hematology analyzer (Diagnostics, Montpellier, France). According to the absolute numbers of granulocytes and lymphocytes, the granulocyte-lymphocyte ratio was recounted.

**PCR analysis.** From 200 μL of EDTA-anti-coagulated blood DNA was isolated using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, in an automatic system for DNA isolation, QIAcube® (Qiagen, Hilden, Germany). Amplification of a fragment (~560 bp) of the 18s rRNA gene was obtained using two pairs of primers in nested PCR. Amplification of a longer fragment (1715 bp) was obtained with the forward primer CRYPTO F 5’-AACCTGGTTGATCCTGCCAGTAGTCAT-3’ and the reverse primer CRYPTO G 5’-GAATGATCCTTCCGCAGGTTCACCTAC-3’. Amplified DNA was subjected to capillary electrophoresis by QIAExcel (Qiagen, Hilden, Germany) and viewed under a UV light. PCR products were purified using a ExoSAP-IT® CLEAN UP (USB, USA) according to the manufacturer’s instructions, and fully sequenced using an ABI PRISM® BigDyeTM Terminator system and GeneAmp® PCR 2400 (PE Biosystems, USA) with forward primer BAB F 5’- CCCTTCATCGGTGGTAACTT-3’ and reverse primer BAB R 5’-GTGGCCACACCCTCGGCTGCC-3’. Sequences were assembled using Lasergene® software (DNASTAR, Madison WI, USA), together with the pertaining SeqMan™ and EditSeq™ software. The sequences obtained were compared within the Gen Bank procurable sequences, in order to identify the Babesia species. In the course of this research, blood samples for hematology, biochemistry and cytokine concentration measurement were taken 1 day and 6 days after the confirmation of babesiosis and antibabesial treatment.

**Biochemical analysis.** Samples for serum biochemistry and cytokine concentrations were collected into gel separator tubes and centrifuged at 1200 x g prior to biochemical analysis. Serum creatinine, alanine aminotranspherase (ALT), alkaline phosphatase (AP), creatine phosphokinase (CPK), glucose and serum bilirubin were determined using biochemical autoanalyser Olympus AU 600 (Olympus Diagnostica, Hamburg). Analyses were performed using standard methods and the manufacturer’s original reagents (Olympus Diagnostica, Hamburg).
Chemokine levels determination. The serum samples for cytokine concentration were separated, and frozen at -80 °C until analysis. Samples were then batch analyzed. Prior to the analyses, the samples were thawed completely, mixed well by vortexing and centrifuged again at 1000× g for 10 minutes to remove particulates. Serum IL-8 and KC chemokine activity was assessed by a canine-specific multiplex assay (CCYTO-90K, Millipore, Billerica, MA) with an automated analyzer (Luminex 200, Luminex Corporation, Austin, TX). The analytes were measured in duplicate, according to the manufacturer’s instructions, with internal quality control. Intra-assay and inter-assay coefficients of variation, as well as minimum detectable concentrations can be viewed online at http://www.millipore.com. The canine cytokine standard was used in the test. Briefly, the plate was pre-wet with wash buffer, that was removed by vacuum. Standards, controls and samples were added to the appropriate wells. After washing, the detection antibodies were added to each well. After incubation, the plate was run on Luminex. The median fluorescent intensity (MFI) was analyzed and the calculation of cytokine and chemokine concentrations was performed using the standard curve. Intra-assay precision for KC-like was 11.5 % and for IL-8 15.6 %, while inter-assay precision was 18.4 % for KC and 15.2 % for IL-8. Minimum detectable concentrations (assay sensitivities) were 1.6 pg/mL for KC and 20.3 pg/mL for IL-8. The mean percent recovery test for KC and IL-8 was 98 %.

Statistical analysis. Statistical analysis was performed using the commercial statistical software, STATISTICA version 10 (StatSoft.Inc., 2011). Comparison of serum chemokine concentrations between healthy dogs and dogs with babesiosis, as well as between dogs with an uncomplicated form and dogs with a complicated form of babesiosis, was performed using the Mann-Whitney U test. P-values of <0.05 were considered to be significant. Correlation of cytokine concentrations and hematological results was assessed by Spearman’s correlation coefficient.

Results
Serum, blood samples and clinical data from 47 dogs were retrospectively included in this study. Among them 16 were healthy dogs pretreated with an antibabesial drug for prophylactic purposes. Babesiosis was diagnosed in 31 dogs.

The study included more than 10 different breeds, and 42 % of them were mixed breed dogs. In the study population there were 58 % males and 42 % females, aged 1-14 years.

No statistical differences (P>0.05) were confirmed between healthy dogs prior to and post antibabesial treatment in serum chemokine concentrations, or in hematological parameters (Table 1).
Table 1. Haematological parameters and serum concentration of chemokines in healthy dogs prior and post antibabesial treatment

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Healthy BI (n = 16)</th>
<th>Healthy AI (n = 16)</th>
<th>Mann-Whitney U P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>10^9/L</td>
<td>9.9 (8.2-13.8)</td>
<td>12.4 (7.7-14.8)</td>
<td>0.3184</td>
</tr>
<tr>
<td>Platelets</td>
<td>10^9/L</td>
<td>243.5 (195-348)</td>
<td>249.5 (193-441)</td>
<td>0.7525</td>
</tr>
<tr>
<td>HMT</td>
<td>%</td>
<td>52.0 (43-55)</td>
<td>51.5 (46-56)</td>
<td>0.8748</td>
</tr>
<tr>
<td>RBC</td>
<td>10^12/L</td>
<td>7.4 (6.7-8.0)</td>
<td>7.2 (6.9-7.6)</td>
<td>0.4308</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>%</td>
<td>27 (20-24)</td>
<td>35 (20-39)</td>
<td>0.0520</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>%</td>
<td>65 (58-70)</td>
<td>59 (58-75)</td>
<td>0.0660</td>
</tr>
<tr>
<td>Band Neutrophils</td>
<td>%</td>
<td>0</td>
<td>0 (0-1)</td>
<td>0.7131</td>
</tr>
<tr>
<td>Monocytes</td>
<td>%</td>
<td>3 (1-5)</td>
<td>1.5 (0-4)</td>
<td>0.1278</td>
</tr>
<tr>
<td>Eosinophiles</td>
<td>%</td>
<td>4 (1-9)</td>
<td>3.5 (1-9)</td>
<td>0.9163</td>
</tr>
<tr>
<td>Granulocytes / lymphocytes</td>
<td></td>
<td>2.4 (1.7-3.5)</td>
<td>1.65 (1.4-3.7)</td>
<td>0.0560</td>
</tr>
<tr>
<td>KC</td>
<td>pg/mL</td>
<td>35.3 (28.7-69.1)</td>
<td>43.1 (27.6-51.7)</td>
<td>0.7131</td>
</tr>
<tr>
<td>IL-8</td>
<td>pg/mL</td>
<td>138.7 (122.1-229.2)</td>
<td>138.7 (130.5-235.1)</td>
<td>0.1892</td>
</tr>
</tbody>
</table>

Haematological parameters and serum concentration of interleukin 8 (IL-8) and keratinocyte chemoattractant (KC) median values (range) in healthy dogs before (BI) and 24 hours after (AI) prophylactic application of imidocarb dipropionate (6.6 mg/kg s.c.). No statistical significant differences were found between prior and after antibabesial treatment (P >0.05, Mann-Whitney U-test).

Table 2. Haematological parameters and serum concentration of chemokines in healthy dogs and dogs with babesiosis 1st day after admission.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Healthy (n = 16)</th>
<th>Babesiosis day 1 (n = 31)</th>
<th>Mann-Whitney U P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>10^9/L</td>
<td>12.4 (7.7-14.8)</td>
<td>6.7 (1.6-32.2)</td>
<td>0.0320</td>
</tr>
<tr>
<td>Platelets</td>
<td>10^9/L</td>
<td>249.5 (193-441)</td>
<td>39.0 (2.0-188.0)</td>
<td>0.0000</td>
</tr>
<tr>
<td>HMT</td>
<td>%</td>
<td>51.5 (46-56)</td>
<td>32.0 (11-46)</td>
<td>0.0020</td>
</tr>
<tr>
<td>RBC</td>
<td>10^12/L</td>
<td>7.2 (6.9-7.6)</td>
<td>4.7 (1.6-6.5)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Granulocytes / lymphocytes</td>
<td></td>
<td>1.65 (1.4-3.7)</td>
<td>4.2 (0.9-99.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>KC</td>
<td>pg/mL</td>
<td>43.1 (27.6-51.7)</td>
<td>531.6 (103.9-2739.3)</td>
<td>0.0000</td>
</tr>
<tr>
<td>IL-8</td>
<td>pg/mL</td>
<td>138.7 (130.5-235.1)</td>
<td>856.3 (100.0-6501.8)</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Haematological parameters and serum concentration of interleukin 8 (IL-8) and keratinocyte chemoattractant (KC) median values (range) in healthy dogs 24 hours after antibabesial treatment and dogs diagnosed with babesiosis 1st day after admission. Statistically significant difference in serum concentration of KC and IL-8, and haematological parameters between dogs with babesiosis on the 1st day of admission and healthy dogs was found (P <0.05, Mann-Whitney U-test).
I. Mayer et al.: Serum levels of chemokines KC and IL-8 in dogs naturally infected with Babesia canis canis

Table 3. Haematological parameters and serum concentration of chemokines in healthy dogs and dogs with babesiosis on the 6th day after admission

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Healthy (n = 16)</th>
<th>Babesiosis day 6 (n = 31)</th>
<th>Mann-Whitney U P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>10⁹/L</td>
<td>12.4 (7.7-14.8)</td>
<td>12.5 (1.0-45.0)</td>
<td>0.5184</td>
</tr>
<tr>
<td>Platelets</td>
<td>10⁹/L</td>
<td>249.5 (193-441)</td>
<td>306.0 (42.0-734.0)</td>
<td>0.1594</td>
</tr>
<tr>
<td>HMT %</td>
<td></td>
<td>51.5 (46-56)</td>
<td>40.5 (17.0-59.0)</td>
<td>0.0000</td>
</tr>
<tr>
<td>RBC 10¹²/L</td>
<td></td>
<td>7.2 (6.9-7.6)</td>
<td>5.8 (2.6-8.8)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Granulocytes / lymphocytes</td>
<td></td>
<td>1.65 (1.4-3.7)</td>
<td>2.5 (0.1-44.0)</td>
<td>0.2536</td>
</tr>
<tr>
<td>KC pg/mL</td>
<td></td>
<td>43.1 (27.6-51.7)</td>
<td>561.2 (110.5-2183.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-8 pg/mL</td>
<td></td>
<td>138.7 (130.5-235.1)</td>
<td>2753.7 (425.2-8818.5)</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Haematological parameters and serum concentration of interleukin 8 (IL-8) and keratinocyte chemoattractant (KC) median values (range) in healthy dogs and dogs diagnosed with babesiosis six days after admission. Six days after admission significantly lower RBC count as well as significantly higher serum concentration of chemokines in dogs with babesiosis was still presented (P <0.05, Mann-Whitney U-test). No statistical significant differences were found for other haematological parameters (P>0.05, Mann-Whitney U-test).

Table 4. Haematological parameters and serum concentration of chemokines in dogs with uncomplicated form and dogs with complicated form of babesiosis 1st day after admission

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Uncomplicated babesiosis day 1 (n = 13)</th>
<th>Complicated babesiosis day 1 (n = 18)</th>
<th>Mann-Whitney U P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>10⁹/L</td>
<td>5.0 (2.5-10.7)</td>
<td>9.3 (1.6-32.2)</td>
<td>0.2887</td>
</tr>
<tr>
<td>Platelets</td>
<td>10⁹/L</td>
<td>39.0 (11.0-68.0)</td>
<td>49.0 (2.0-188.0)</td>
<td>0.1864</td>
</tr>
<tr>
<td>HMT %</td>
<td></td>
<td>36.0 (11.0-46.0)</td>
<td>30.5 (16.0-41.0)</td>
<td>0.1385</td>
</tr>
<tr>
<td>RBC 10¹²/L</td>
<td></td>
<td>5.5 (1.6-6.3)</td>
<td>4.6 (2.12-6.6)</td>
<td>0.2297</td>
</tr>
<tr>
<td>Granulocytes / lymphocytes</td>
<td></td>
<td>2.7 (0.9-23.5)</td>
<td>4.2 (2.3-99.0)</td>
<td>0.4711</td>
</tr>
<tr>
<td>KC pg/mL</td>
<td></td>
<td>329.9 (103.9-968.9)</td>
<td>567.0 (162.0-2739.3)</td>
<td>0.0322</td>
</tr>
<tr>
<td>IL-8 pg/mL</td>
<td></td>
<td>600.7 (349.0-5018.1)</td>
<td>907.5 (100.3-5501.1)</td>
<td>0.5089</td>
</tr>
</tbody>
</table>

Haematological parameters and serum concentration of interleukin 8 (IL-8) and keratinocyte chemoattractant (KC) median values (range) in dogs diagnosed with uncomplicated babesiosis and dogs with complicated babesiosis on the 1st day after admission. Statistically significant difference in serum concentration of KC was found (P<0.05, Mann-Whitney U-test). There was no statistically significant difference for other investigated parameters (P>0.05, Mann-Whitney U-test).
Table 5. Haematological parameters and serum concentration of chemokines in dogs with uncomplicated and dogs with complicated babesiosis on the 6th day after admission

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Uncomplicated babesiosis day 6 (n = 13)</th>
<th>Complicated babesiosis day 6 (n = 18)</th>
<th>Mann-Whitney U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>10^9/L</td>
<td>11.9 (6.0-19.1)</td>
<td>15.9 (1.0-45.0)</td>
<td>0.1804</td>
</tr>
<tr>
<td>Platelets</td>
<td>10^3/L</td>
<td>316.0 (110-397)</td>
<td>294.0 (42.0-734.0)</td>
<td>0.6303</td>
</tr>
<tr>
<td>HMT</td>
<td>%</td>
<td>45.0 (33.0-59.0)</td>
<td>37.0 (17.0-50.0)</td>
<td>0.0040</td>
</tr>
<tr>
<td>RBC</td>
<td>10^12/L</td>
<td>6.6 (4.5-8.8)</td>
<td>5.1 (2.6-7.0)</td>
<td>0.0050</td>
</tr>
<tr>
<td>Granulocytes/lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>pg/mL</td>
<td>440.0 (117.1-2009.4)</td>
<td>830.1 (110.5-2183.7)</td>
<td>0.0445</td>
</tr>
<tr>
<td>IL-8</td>
<td>pg/mL</td>
<td>2852.0 (1391.2-6293.8)</td>
<td>1910.8 (425.4-8818.5)</td>
<td>0.6008</td>
</tr>
</tbody>
</table>

Haematological parameters and serum concentration of interleukin 8 (IL-8) and keratinocyte chemoattractant (KC) median values (range) in dogs diagnosed with uncomplicated babesiosis and dogs with complicated babesiosis six days after admission. Six day after admission in dogs with complicated babesiosis significantly lower RBC count and significantly higher granulocyte-lymphocyte as well as significantly higher serum concentration of KC (P<0.05, Mann-Whitney U-test). There was no statistically significant difference in IL-8 concentration between dogs with complicated and dogs with uncomplicated babesiosis (P>0.05, Mann-Whitney U-test)

Microscopic evaluation of blood smears demonstrated the presence of *B. canis* in the erythrocytes of all 31 sick dogs. Sequence analysis confirmed the presence of *B. canis* in all 31 samples tested.

Out of the 31 dogs with babesiosis, in 18 dogs a complicated form and in 13 an uncomplicated form of babesiosis was diagnosed.

Serum concentrations of KC and IL-8 were significantly increased (P<0.05) in dogs with babesiosis, when compared with healthy controls on both the 1st and 6th days of research (Table 2, Table 3). Six days after admission, the serum concentration of IL-8 was significantly higher (P<0.05) compared to the concentration of IL-8 measured 1 day after admission (Table 3).

Twenty-four hours after admission, hematological results in dogs with babesiosis showed significantly lower numbers of leukocytes, RBC, and platelets, as well as a significantly higher granulocyte-lymphocyte ratio, compared with healthy controls (Table 2). Six days after admission there was still a significantly lower RBC count in dogs with babesiosis (Table 3).

The serum concentration of KC in dogs with an uncomplicated form of babesiosis 24 hours after admission was significantly higher compared to the KC concentration in dogs with complicated babesiosis. Six days after admission, the RBC count and granulocyte-lymphocyte ratio were significantly lower, whereas serum concentration of KC was significantly higher in dogs with complicated babesiosis, compared to dogs with
an uncomplicated form of the disease. There was no statistically significant difference in IL-8 concentrations between dogs with complicated and dogs with uncomplicated babesiosis (Table 4, Table 5)

A statistically significant correlation between concentrations of chemokines and hematological parameters was established in dogs with babesiosis. At admission, a significant negative correlation between KC concentration and HCT ($R = -0.341$, $P<0.05$), as well as a significant positive correlation between KC and platelet count ($R = 0.415$, $P<0.05$) were found. There was no statistically significant correlation between hematological parameters and IL-8 concentration.

Six days after admission a significant negative correlation between KC concentration and hematocrit value ($R = -0.672$, $P<0.05$), as well as a significant positive correlation between KC concentration and WBC count ($R = 0.589$, $P<0.05$) and the granulocyte-lymphocyte ($R = 0.464$ $P<0.05$) ratio were found. The concentration of IL-8 correlated significantly positively with the granulocyte-lymphocyte ratio ($R = 0.459$, $P<0.05$)

**Discussion**

Recent research related to inflammatory factor activity in dogs suffering from babesiosis has led to the conclusion that the inflammatory response of the host plays a significant role in the pathogenesis of uncomplicated and complicated forms of babesiosis. The clinical manifestation and outcome of babesiosis depends on the balance of systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response (CARS). The shift in the balance in favour of the production of pro-inflammatory factors results in the development of sepsis and septic shock. Consequently this leads to a negative outcome of the disease. Pro-inflammatory and anti-inflammatory responses are determined by the interaction of various cytokines.

The production and activity of cytokines are closely related to the cells of the haematopoietic system, whether in terms of the production of cytokines themselves or in terms of the influence of individual cytokines on the production, migration and maturation of blood cells (OPPENHEIM et al., 2000).

This is the first clinical study in which KC and IL-8 concentrations in dogs with *B. canis canis* infection have been measured. In our study, elevated levels of IL-8 and KC concentrations were found in dogs with babesiosis compared to healthy controls, as well as higher concentrations of chemokines in dogs with a complicated form of babesiosis. These results are consistent with the results of other studies investigating the role of KC and IL-8 in the pathogenesis of inflammatory diseases. Increased concentrations of KC and IL-8 have been demonstrated in other systemic inflammatory diseases, such as: ehrlichiosis, anaplasmosis, leishmaniasis, and hepatozoonosis infections (TAJIMA and RIKIHISA, 2005; ALVES et al., 2009).
Overproduction of IL-8 is also associated with several pathological conditions, such as: spirocercosis, *Helicobacter* spp., osteosarcoma, gastro-oesophageal reflux disease, leptospiral and hantaviral infections (WIINBERG et al., 2005; PAOLONI et al., 2009; RIEDER et al., 2010; ŽMAK et al., 2011; DVIR et al., 2012).

It is known that *B. canis* infection is also characterized by systemic inflammatory response (MATIJATKO et al., 2007). The elevated levels of IL-8 and KC concentrations in dogs with babesiosis compared to healthy controls, as well as higher concentrations of chemokines in dogs with a complicated form of babesiosis found in our study could be considered to be further evidence that the systemic inflammatory reaction has an important role in the pathogenesis of canine babesiosis. The positive correlation between KC-like and IL-8 levels, the total number of leukocytes, and the granulocyte to lymphocyte ratio in our results may be explained by the biological activity of the investigated chemokines. KC and IL-8 belong to the group of CXC chemokines which perform their biological activity by binding to CXCR1 and CXCR2 chemokine receptors. The basic biological activity of these chemokines is the activation and chemotaxis of neutrophils. It has been shown that KC also functions as a mediator of MCP-1 activity (LOCATI et al., 2005; FRINK et al., 2007). Our results clearly demonstrate that IL-8 and KC-like levels are also closely related to a reduced number of erythrocytes.

Similar results were obtained in research conducted by KJELGAARD-HANSEN et al. (2011). It is necessary to emphasize that the above mentioned research is, to the best of our knowledge, the only research dealing with serum chemokine levels in dogs suffering from a systemic, acute inflammatory haemolytic disease published so far.

An elevated concentration of KC and IL-8 in dogs with immune mediated hemolytic anemia was detected in this study. Compared to our results, dogs with IMHA exhibited considerably higher serum IL-8 and KC-like levels. At the same time, dogs that developed IMHA had significantly more severe anemia than dogs with babesiosis in our study. The precise interaction between chemokines and erythrocytes has still not been completely explained.

Recent research conducted on cell cultures has proven that the enhanced production of CXC chemokines may trigger oxidative stress (BECK et al., 2001; FALEIROS et al., 2009) which, besides secondary IMHA, has been considered to be the main cause of erythrocyte destruction in canine babesiosis (IRWIN and HUTCHINSON, 1991; CRNOGAJ et al., 2010).

It has been presumed that neutrophils, triggered by the activity of anti-inflammatory chemokines, recognize erythrocytes damaged by the parasite, the immune complexes and the parasites themselves. This results in the activation of phagocytes and leads to their release into extracellular space. The presence of phagocytes in the extracellular space may produce a negative paracrine effect on the erythrocytes. Oxidation/nitrosylation of membrane lipids and proteins causes structural damage to the erythrocyte membrane.
I. Mayer et al.: Serum levels of chemokines KC and IL-8 in dogs naturally infected with *Babesia canis canis* and erythrocyte lysis. The destroyed erythrocytes additionally release hemoglobin and its constituents as well as oxidized lipid molecules, such as malondialdehydes. Elevated levels of these molecules in the blood, urine or other body fluids are considered to be direct indicators of oxidative stress (MARKS et al., 1996; GRAÇA-SUOZA et al., 2002).

A positive correlation between concentrations of malondialdehydes and the level of anemia in dogs with babesiosis was also established, confirming the role of oxidative stress in the pathogenesis of babesiosis (CRNOGAJ et al., 2010). The aforementioned presumed interaction between erythrocytes and the CXC chemokines, as well as the effect of oxidative stress on erythrocytes and chemokine production could explain the significantly higher serum level of chemokines in dogs suffering from IMHA, compared to the levels measured in dogs with babesiosis in our study.

Although the correlation between IL-8 and KC-like levels and the level of anemia in dogs should be explored in more detail, a partial explanation can be provided by research results in human medicine. Those studies showed that human erythrocytes have expressed surface antigens by which they bind CXC chemokines. In that way, erythrocytes regulate their bioavailability (DARBONNE et al., 1991; LEE et al., 2006; MANGALMURTI et al., 2008). Those results raise the question whether canine erythrocytes function in a similar manner.

Our finding of a statistically relevant negative correlation between KC levels, hematocrit values and the erythrocyte count in dogs with babesiosis could be considered as evidence of the potential interplay between chemokines and erythrocytes. Such a correlation was established on both the 1st and 6th days of the research, in dogs suffering from complicated babesiosis. This result confirms the existence of a close connection between KC activity, systemic inflammatory reaction and hemolysis.

Although numerous studies have confirmed the relevance of the systemic inflammatory response in the pathogenesis of canine babesiosis, the activity of individual chemokines as well as the connection between chemokine levels, clinical features and the severity of the disease, have not been investigated so far.

The results of this research prove that there is an indisputable connection between IL-8 and KC levels and the development of anemia, leukocytosis and complicated babesiosis. The chemokines investigated in this study are defined as significant inflammatory mediators, so the connection described could be considered as further evidence that the pathogenesis of babesiosis is based primarily on systemic inflammatory reaction.

**Acknowledgements**

This research was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (Project No. 053-0532266-2220).
I. Mayer et al.: Serum levels of chemokines KC and IL-8 in dogs naturally infected with *Babesia canis canis*

**References**


I. Mayer et al.: Serum levels of chemokines KC and IL-8 in dogs naturally infected with Babesia canis canis


I. Mayer et al.: Serum levels of chemokines KC and IL-8 in dogs naturally infected with *Babesia canis canis*


MATIJATKO, V. (2003): Acute phase reactants in dogs naturally infected with hemoprotozoon *Babesia canis* treated with imidocarb dipropionate. Doctoral thesis. Faculty of Veterinary Medicine, University of Zagreb, Croatia.


I. Mayer et al.: Serum levels of chemokines KC and IL-8 in dogs naturally infected with *Babesia canis canis*


Received: 2 April 2014
Accepted: 13 May 2015


**SAŽETAK**

Babezioza pasa uzrokovana vrstom *Babesia canis canis* jedna je od najčešćih bolesti pasa u Hrvatskoj. Poznato je da je sustavni upalni odgovor (SIRS), koji se smatra osnovom patofiziološkog procesa tijekom bezbioze, reguliran međudjelovanjem niza proupalnih i protuupa lnih citokina. Cilj ovog istraživanja bio je odrediti koncentraciju CXC kemokina: kemotraktanta za keratinocite (KC) i interleukina 8 (IL-8) u serumu pasa oboljelih od bezbioze te usporedivati koncentraciju ovih citokina u serumu zdravih pasa. Također je željno ustanoviti je li je aktivnost KC i IL-8 povezana s razvojem komplikiranog oblika bezbioze i ishodom bolesti. Koncentracija kemokina u serumu određivana je Luminex xMap tehnologijom komercijalnim multiplex komplemetom specifičnim za pse. Dobiveni rezultati pokazali su da je aktivnost KC povezana s razvojem anemije i leukocitoze, dok koncentracija IL-8 pozitivno korelira s omjerom granulocita i limfocita. Viša koncentracija obaju kemokina povezana je s razvojem komplikiranog oblika bolesti. Stoga se koncentracija KC i IL-8 u serumu može smatrati važnim čimbenikom u patogenezi bezbioze pasa.

**Ključne riječi:** kemotraktant za keratinocite, IL-8, upalni odgovor, pas, bezbioza