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# Antioxidant status, and blood zinc and copper concentrations in dogs with uncomplicated babesiosis due to *Babesia canis* infections

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## Abstract

**Introduction:** The aim of the study was to demonstrate a link between uncomplicated *Babesia canis* infection in dogs and blood concentrations of zinc and copper and erythrocytic antioxidant defence – activities of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). **Material and Methods:** The study was based on 15 naturally occurring cases of canine babesiosis with anorexia, pyrexia, depression, pale mucous membrane, splenomegaly and dark red urine. Microscopic examination of Giemsa-stained peripheral blood smears and the results of PCR confirmed *B. canis* infection. Seven apparently healthy dogs brought in for either a check-up or vaccination were used for comparison. **Results:** The levels of the erythrocytic antioxidant enzymes - SOD and CAT - were significantly higher in the infected dogs than in cytologically negative dogs. The levels of blood micronutrients were significantly lower in the infected dogs (0.478 µg of zinc per mL *vs* 1.241 µg/mL and 0.722 µg of copper per mL *vs* 1.392 µg/mL). **Conclusion:** Oxidative stress can be posited as one of the mechanisms leading to anaemia in dogs with babesiosis, and therefore antioxidant biomarker and copper and zinc concentrations could be used as indicators of disease severity and prognostic markers.

**Keywords:** *Babesia canis*, copper, zinc, superoxide dismutase, catalase.

## Introduction

Canine babesiosis is a common and clinically significant tick-borne disease caused by haematozoan parasites of the genus *Babesia* (4). Two morphologically distinct forms of the erythrocytic stage in the canine host were recognised – the larger form (3–5 µm) *B. canis* and the smaller (1–3 µm) *B. gibsoni* (3). Cross-immunity, serological testing, vector specificity and molecular phylogeny were used to reclassify *Babesia canis* into three separate species (*B. canis*, *B. rossi* and *B. vogeli*) (10, 33). Within the small piroplasms, three distinct species are recognised as causing disease in dogs: *B. gibsoni*, *B. conradae*, and *B. vulpes* (*B. microti*-like piroplasm, *Theileria annae*) (17, 18). Recently, Mierzejewska *et al.* (21) detected *Babesia vulpes* in foxes in Poland – this is a new species, which can cause infestations in the country's dogs.

Our earlier study described the clinical course of babesiosis in dogs from areas of eastern Poland and selected aberrant haematological and biochemical parameters of the serum of dogs infected with this disease (4). The clinical picture of infection with *B. canis* is diverse, ranging from hyper-acute through acute to chronic disease (4).

*Babesia* protozoa invade the erythrocytes of the host animals, and the destruction of the parasitised erythrocytes is a common consequence of infection, leading to anaemia (4). The severity of the anaemia is not always proportional to the degree of parasitaemia (8). The quantity of destroyed erythrocytes is usually much higher than the degree of parasitaemia, suggesting

that non-parasitised erythrocytes may also be damaged (11). Some of the proposed mechanisms responsible for this phenomenon are sequestration of infected erythrocytes in microcirculation, decreased erythrocyte deformability, and haemodilution and destruction of red blood cells due to the effects of oxidative stress (11, 23, 27, 28, 29). Free radicals and other reactive oxygen species (ROS) have been implicated as playing an important role in tissue damage in a variety of pathological processes (8). Overproduction of ROS in diverse pathological conditions leads to oxidative damage to macromolecules, which results in more intensive lipid peroxidation and DNA strand breaks (16, 22). To counteract the oxidative damage caused by ROS generated during infections, a multi-layered defence system is generated, including DNA repair systems, scavenging substrates and the antioxidant enzyme system (7, 11). Indirect loss of essential body nutrients caused by accelerated metabolism or consumption has been speculated to occur during the course of infectious diseases (8). The plasma zinc value appears susceptible to shock and fever (8). Zinc deficiency has also been reported as enhancing oxidative damage to proteins, lipids and DNA in rat tissues (22). Micronutrients such as zinc and copper are also essential components of the body's antioxidant defence and play an important role in the prevention of free radical–induced damage to tissues required for the maintenance of health and production (15). Zinc and copper are utilised for the synthesis of the important antioxidant enzyme, Cu–Zn superoxide dismutase (SOD), which catalyses the conversion of the superoxide radical to the less oxidising $H_2O_2$.

The aim of the study was to demonstrate a link between uncomplicated *B. canis* infection in dogs and blood concentrations of zinc and copper and erythrocytic antioxidant defence.

## Material and Methods

**Animals used in the study.** Fifteen dogs with babesiosis (group 1) aged 2-7 years (mean 4.13) and seven healthy dogs (group 2) aged 1-5 years (mean 3.29) were studied prospectively between March and June 2020. The 15 dogs in group 1 showed symptoms of uncomplicated babesiosis, which appeared 2-3 days before their admission to the clinic. The seven healthy dogs in group 2 were admitted to the clinic for a routine check-up. They did not show any clinical abnormalities. Blood was collected from all dogs for haematological, biochemical and molecular analyses.

**Blood analyses.** The samples were collected in a routine way, before any treatment was administered. Blood was taken from the cephalic vein into tubes with ethylenediaminetetraacetic acid (EDTA) for haematological and PCR evaluation, and into plain tubes for biochemical evaluation. Thin blood smears were stained by the Giemsa method and examined for parasites.

The analyses of Zn and Cu trace elements levels were carried out using inductively coupled plasma-optical emission spectrometry (ICP-OES; iCAP 6000 series, Thermo Fisher Scientific, Waltham, MA, USA). Each measurement was performed three times and averages were used for the analysis. In the study, the appropriate wavelengths of the Zn and Cu elements (206.200 nm and 324.754 nm, respectively) were used for analysis in the ICP-OES device. Stock solutions for Zn and Cu trace elements were prepared from standard solutions (Chem-Lab NV, Zedelgem, Belgium) and distilled water was used as a blank solution. Calibration graphs were obtained from the ICP-OES device using blank and standard solutions and the concentration measurements of Zn and Cu trace elements were carried out according to the graphs. The blood plasma samples were diluted with distilled water. The standard solutions were measured again for system control after every five sample analyses. The concentration levels of Zn and Cu were expressed as μg/mL.

The activities of glutathione (GSH), SOD and catalase (CAT) were determined in whole blood in tubes with lithium heparin. Superoxide dismutase activity in the erythrocytes was determined using a commercial Ransod diagnostics kit (Randox Laboratories, Crumlin, UK), according to the manufacturer's instructions, and CAT activity was determined according to Aebi (5). The plasma content of GSH was determined according to the methods described by Bartosz (6).

**Molecular examination.** DNA for PCR was extracted from EDTA-anticoagulated whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The amplification of *B. canis* DNA was performed using the forward primer BAB GF2 (5′-GTC TTG TAA TTG GAA TGA TGG-3′) and the reverse primer BAB GR2 (5′-CCA AAG ACT TTG ATT TCT CTC-3′), which duplicate a 559-bp region of the 18S rRNA gene of *B. canis* (2).

The real-time PCR reaction for all the isolated DNA samples was carried out using the Rotor-Gene RG6000 real-time DNA amplification system (Corbett Research, Mortlake, NSW, Australia) with SYBR Green 1 dye in thin-walled test-tubes with a capacity of 100 μL. A DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland ) was used to conduct the high-specificity reaction.

The reaction mixture, with a capacity of 20 μL, consisted of the following components: 2 μL of the DNA matrix, 7.2 μL of water, 0.4 μL of each of the GF2 and GR2 primers (final concentration of 50 pM), 10 μL of Master Mix containing a hot start version of the modified *Thermus brockianus* (Tbr) polymerase, buffer for the Tbr polymerase, deoxynucleotide triphosphate, $MgCl_2$, and the intercalating SYBR Green 1 dye.

The optimised real-time PCR reaction included 50 cycles, each comprising three stages: denaturation at 92°C for 60 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s (3).

The PCR products were then purified using QIAquick spin columns (Qiagen), eluted in 50 µL of Tris 10 mM, pH 7.6, and sequenced at the Research Institute of the Polish Academy of Sciences, in Warsaw. DNA sequences were assembled and edited using SeqMan (Lasergene, DNAStar, Madison, WI, USA), and ClustalV alignments were generated to the published *B. canis canis* 18S rRNA gene (GenBank accession numbers EU622792 and EU622793).

**Statistical analysis.** The Mann-Whitney rank test was used to demonstrate the differences in Zn and Cu concentrations between the groups and between other haematological and biochemical parameters. Changes were considered statistically significant at $P < 0.05$. Statistica 10.0 PL software (StatSoft, now Tibco, Palo Alto, CA, USA) was used for the calculations.

## Results

All dogs in group 1 were apathetic. They also showed signs of anorexia, pyrexia, depression, and splenomegaly and had pale mucous membranes and dark red urine. The dogs in group 1 were injected subcutaneously with imidocarb dipropionate solution (Imizol; Schering Plough Animal Health, now MSD Animal Health, Kenilworth, NJ, USA), in a dosage of 5 mg/kg b.w., which resulted in recovery in all cases.

None of the dogs in group 2 showed any clinical signs of the disease. They were clinically healthy for the whole period of the study.

The results of haematological analysis showed some abnormally low levels in group 1 dogs but none in group 2 animals. A drop in haematocrit below 37% (the lower limit of normal) was found in every dog in group 1. A decrease of erythrocytes below $5.5 \times 10^{12}$ (the lower limit of normal) was noted in 9 of the dogs with uncomplicated babesiosis. Leukopaenia (white blood cell count $< 6 \times 10^9$) occurred in 6 sick dogs, while thrombocytopenia (platelet count $< 200 \times 10^9$) was reported in all afflicted dogs (Table 1).

Copper concentration was below normal in 14 out of the 15 dogs in group 1 (mean value 0.722 µg/mL) and was statistically significantly lower ($P = 0.000247$) than in the dogs in group 2, which all showed normal blood copper concentrations (mean value 1.392 µg/mL). The majority of the dogs in group 1 (13/15) had zinc blood concentrations below the lower limit of normal (mean value 0.478 µg/mL), whereas all the dogs in group 2 had normal values for this parameter (mean value 1.241 µg/mL). The differences were statistically significant ($P = 0.000323$) (Tables 2 and 3).

Erythrocytic SOD activity in *B. canis*-infected dogs was significantly higher ($P = 0.000421$) than in the control dogs. The mean value of SOD in group 1 was 36.78 U/mL, while in group 2 it was 31.08 U/mL.

**Table 1.** Results of haematological examinations of dogs from group 1 (dogs with babesiosis) and 2 (healthy dogs)

| Group | Dog | WBC ($\times 10^9$) | RBC ($\times 10^{12}$) | Ht (%) | PLT ($\times 10^9$) |
|---|---|---|---|---|---|
| 1 | 1 | 6.8 | 6.76 | 38.7 | 43 |
| | 2 | 5.0 | 7.29 | 45.0 | 28 |
| | 3 | 6.9 | 6.20 | 38.4 | 24 |
| | 4 | 4.3 | 6.54 | 40.5 | 27 |
| | 5 | 6.2 | 5.39 | 32.0 | 30 |
| | 6 | 7.8 | 5.98 | 34.4 | 65 |
| | 7 | 10.3 | 6.40 | 39.2 | 35 |
| | 8 | 6.8 | 3.78 | 24.8 | 19 |
| | 9 | 6.1 | 3.97 | 25.2 | 31 |
| | 10 | 9.3 | 4.30 | 25.6 | 39 |
| | 11 | 4.1 | 4.73 | 29.7 | 16 |
| | 12 | 7.7 | 5.08 | 32.4 | 48 |
| | 13 | 5.6 | 4.75 | 29.7 | 28 |
| | 14 | 4.8 | 5.07 | 31.7 | 29 |
| | 15 | 5.5 | 5.11 | 36.2 | 46 |
| 2 | 16 | 10.4 | 6.40 | 42.1 | 231 |
| | 17 | 9.8 | 6.54 | 39.9 | 379 |
| | 18 | 8.9 | 6.86 | 44.4 | 224 |
| | 19 | 8.8 | 7.12 | 49.6 | 443 |
| | 20 | 6.8 | 6.54 | 39.9 | 379 |
| | 21 | 9.9 | 6.16 | 42.1 | 220 |
| | 22 | 8.4 | 6.58 | 42.3 | 328 |
| | Range | 6.0–17.0 | 5.50–8.50 | 37.0–55.0 | 200–500 |

WBC – white blood cells; RBC – red blood cells; Ht – haematocrit; PLT – platelets

**Table 2.** Cu and Zn concentrations in blood of dogs from groups 1 (dogs with babesiosis) and 2 (healthy dogs)

| Dog | Cu 1.350 µg/mL | Zn 1.485 µg/mL | Group |
|---|---|---|---|
| 1 | 0.788 | 0.400 | 1 |
| 2 | 0.713 | 0.463 | 1 |
| 3 | 0.578 | 0.249 | 1 |
| 4 | 0.650 | 0.379 | 1 |
| 5 | 0.818 | 0.455 | 1 |
| 6 | 0.658 | 0.463 | 1 |
| 7 | 0.524 | 0.404 | 1 |
| 8 | 0.823 | 0.905 | 1 |
| 9 | 0.724 | 0.610 | 1 |
| 10 | 0.662 | 0.466 | 1 |
| 11 | 0.684 | 0.383 | 1 |
| 12 | 0.790 | 0.380 | 1 |
| 13 | 0.812 | 0.622 | 1 |
| 14 | 0.792 | 0.410 | 1 |
| 15 | 0.816 | 0.587 | 1 |
| 16 | 0.894 | 1.412 | 2 |
| 17 | 1.120 | 1.184 | 2 |
| 18 | 1.830 | 0.923 | 2 |
| 19 | 0.993 | 1.340 | 2 |
| 20 | 1.751 | 0.882 | 2 |
| 21 | 1.344 | 1.612 | 2 |
| 22 | 1.812 | 1.332 | 2 |

**Table 3.** CAT, SOD and GSH activity in dogs of groups 1 (dogs with babesiosis) and 2 (healthy dogs)

| Dog | CAT (U/mL) | SOD (U/mL) | GSH (U/mL) | Group |
|---|---|---|---|---|
| 1 | 9.6966 | 38.41 | 3.1 | 1 |
| 2 | 6.2483 | 38.70 | 1.5 | 1 |
| 3 | 7.9172 | 36.34 | 4.5 | 1 |
| 4 | 6.2483 | 33.52 | 3.7 | 1 |
| 5 | 2.6483 | 36.88 | 2.4 | 1 |
| 6 | 4.4690 | 38.16 | 3.4 | 1 |
| 7 | 2.4000 | 36.88 | 4.9 | 1 |
| 8 | 4.8828 | 34.98 | 3.6 | 1 |
| 9 | 3.8207 | 34.75 | 3.9 | 1 |
| 10 | 3.4414 | 34.25 | 5.9 | 1 |
| 11 | 4.2631 | 36.21 | 2.3 | 1 |
| 12 | 3.9343 | 38.18 | 3.1 | 1 |
| 13 | 4.9251 | 39.46 | 2.3 | 1 |
| 14 | 3.2887 | 39.14 | 2.1 | 1 |
| 15 | 6.1181 | 35.82 | 3.2 | 1 |
| 16 | 2.1241 | 34.57 | 3.0 | 2 |
| 17 | 0.8552 | 28.69 | 2.3 | 2 |
| 18 | 1.0000 | 31.51 | 3.6 | 2 |
| 19 | 0.6621 | 30.34 | 2.7 | 2 |
| 20 | 0.7448 | 31.72 | 2.2 | 2 |
| 21 | 1.8342 | 29.23 | 2.9 | 2 |
| 22 | 1.5261 | 31.51 | 4.4 | 2 |

CAT – catalase; SOD – superoxide dismutase; GSH – glutathione

The CAT activity in the erythrocytes of the dogs infected with *B. canis* was also higher than in those of the healthy dogs. The mean value in group 1 was 4.9535 U/mL and in group 2 was 1.2495 U/mL. The differences were statistically significant (P = 0.000246).

There was no significant difference (P = 0.458446) in the GSH activity between the infected and control dogs. The mean value of this parameter in the blood of dogs with babesiosis was 3.26 U/mL, whereas in that of healthy dogs it was 3.01 U/mL.

*B. canis* DNA was detected in the blood samples taken from all 15 infected dogs examined with the real-time PCR. The products were visualised with the electrophoresis method in agarose gel. Their size compared to the mass standard was about 559 bp (Fig. 1). The Ct values read from the amplification curve fluctuated around 17 cycles for all the examined samples (Fig. 2). The melting temperature was between 78°C and 81°C.

Based on the similarities between sequences of the 18S RNA gene fragment, five of the isolated *Babesia* protozoa were classified as the EU622792 18S RNA-A strain and the remaining nine as the EU622793 18S RNA-B strain.

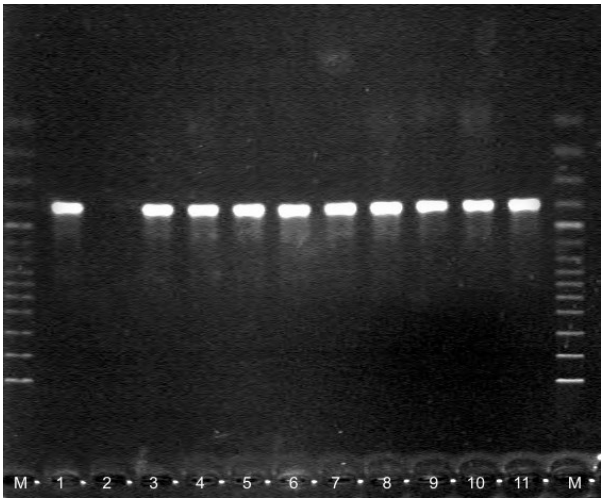


**Fig. 1.** PCR amplification of a partial sequence of *B. canis* 18S RNA gene (product size 559 bp)
M – 100bp molecular weight marker; 1 – positive control; 2 – negative control; 3–11 – studied samples from dogs with babesiosis

## Discussion

The aim of this study was to evaluate the possible changes in three antioxidant biomarkers (SOD, CAT, and GSH), as well as in Cu and Zn concentrations in dogs infected with babesiosis. The literature data on this subject in dogs are very scarce, and can be found in only two studies (8, 11). Dogs naturally infected with *B. canis* and investigated by Crnogaj *et al*. (12) presented serum malondialdehyde levels which confirmed the presence of oxidative stress. Oxidative stress seems to be one of the mechanisms leading to erythrocyte deformation in dogs with babesiosis; therefore, antioxidant biomarkers, as well as copper and zinc, could be used as indicators of anaemia severity.

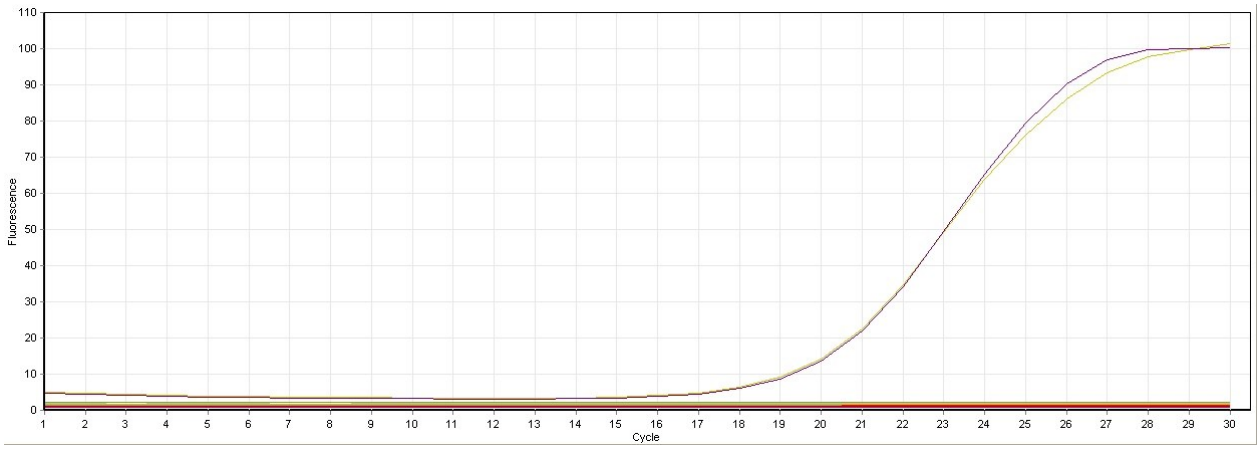


**Fig. 2.** Real-time PCR amplification curve. Ct values fluctuated around 17 cycles for all the examined samples

Our own studies revealed significantly lower concentrations of Zn and Cu in the blood of dogs with babesiosis compared to the control group. In anaemic cattle infected with *B. bigemina*, decreased serum levels of copper and zinc were observed. It can be assumed that the decreased levels of trace elements represent their coordinated antioxidant role along with antioxidant enzyme activities during the infection (14). Both elements play a role in the synthesis of an antioxidant enzyme, Cu–Zn SOD, so their reduced concentrations in infected dogs may be caused by their increased use. On the other hand, in the course of various infectious diseases the levels of these elements in infected dogs are lower (especially in the acute phase of the infection) as a result of increased metabolism and reduced supply of Cu and Zn with food, due to anorexia associated with stress and fever (9, 31). Such mechanisms were observed, for instance, in cattle with theileriosis (19, 20) and in dogs with babesiosis caused by *B. gibsoni* invasion (8).

Our studies demonstrated that in dogs with babesiosis, the SOD and CAT activity was higher than in healthy dogs. These findings conflict with the results presented by Crnogaj *et al.* (11), who observed decreased activity of these antioxidant biomarkers in dogs infected with *B. canis*. Similarly, in sheep with babesiosis (14), in cattle with theileriosis (26) and in people suffering from malaria (13), reductions in SOD, CAT and GSH activity were reported. These enzyme activity profiles may be determined by the disease stage. Our study involved dogs with an early, uncomplicated form of babesiosis. The study by Crnogaj *et al.* (11) demonstrated that SOD, CAT and GSH showed a significantly lower activity in dogs with multiple organ dysfunction syndrome (MODS) than in those with uncomplicated babesiosis. The different stages of the disease might explain the discrepancy between the results.

Our observations are confirmed by the results of studies by Otsuka *et al.* (25) and Chaudhuri *et al.* (8). They demonstrated increased activity of SOD and CAT in dogs infected by *B. gibsoni*, which is consistent with our findings. This may be a defence mechanism, where the organism attempts to prevent the oxidative damage of cells in the course of babesiosis. SOD, CAT and GSH are the major enzymes present in red blood cells to counteract the toxic effects of ROS such as superoxide radicals and hydrogen peroxides (8). The increased activity of SOD and CAT may also be due to a higher number of reticulocytes in the blood of infected animals produced by the bone marrow in response to the destruction of mature erythrocytes by the protozoa (25, 30, 32). In addition, CAT is responsible for the breakdown of $H_2O_2$, an important ROS produced during metabolism, so increased CAT activity might correlate with the generation of $H_2O_2$ in *B. canis*-infected dogs.

We did not observe any correlation between the type of parasite strain causing the infection and the SOD, CAT, and GSH in the infected dogs, as happens with other clinical parameters (1, 4). Further studies in this respect are necessary, conducted on a larger group of animals.

In conclusion, the clinical study demonstrated that in dogs with uncomplicated early babesiosis, contrary to the biomarker picture in animals with advanced disease associated with MODS, the SOD and CAT activity were increased, while concentrations of Cu and Zn were reduced. Considering the strong negative correlation between the activity of antioxidants (SOD, CAT and GSH) in MODS cases and lethal outcome, it appears that the increased activity of SOD and CAT demonstrated in the study is a favourable prognostic, indicative of the uncomplicated form of the disease.

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