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ORIGINAL RESEARCH PAPER

Elevated serum manganese concentration in dogs as a possible predisposing factor of cerebral babesiosis in dogs

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ABSTRACT

The aim of this study was to demonstrate a relationship between the occurrence of clinical signs of brain involvement in dogs with babesiosis and the concentration of manganese (Mn) in their serum. The study included seven dogs with early babesiosis (Group 1), seven dogs with cerebral babesiosis (Group 2) and seven healthy dogs (Group 3). Haematological and biochemical blood tests were performed in all dogs, and the results were analysed statistically. The Mann–Whitney rank test was used to demonstrate the differences in Mn concentrations, as well as other haematological and biochemical parameters between groups. In dogs in Group 2 with cerebral babesiosis, as compared to dogs in Groups 1 and 3, a statistically significant increase in serum Mn concentration was shown ($P = 0.002$ and $P = 0.029$) that may have been associated with the development of anaemia and/or impairment of liver function. Given the well-established neurotoxic effects of Mn in humans, experimental rodents and primates, additional studies on the role of Mn in the pathogenesis of the cerebral form of canine babesiosis are warranted.

KEYWORDS

Babesia canis, cerebral babesiosis, manganese, neurotoxicity

INTRODUCTION

Canine babesiosis is a common and clinically significant tick-borne disease caused by haematozoan parasites of the genus *Babesia* (Adaszek et al., 2009). Two morphologically distinct forms of the erythrocytic stage in the canine host have been recognised: the larger (3–5 μm) *Babesia canis* and the smaller (1–3 μm) *Babesia gibsoni* (Adaszek and Winiarczyk, 2010; Parsh and Whitney, 2020). Cross-immunity, serological testing, vector specificity and molecular phylogeny were used to reclassify *B. canis* into three separate species (*B. canis*, *Babesia rossi* and *Babesia vogeli*) (Zahler et al., 1998; Costa-Júnior et al., 2009). Within the small piroplasms, three distinct species are recognised to cause disease in dogs: *B. gibsoni*, *Babesia conradae* and *Babesia microti*-like piroplasm (*Theileria annae*) (Kjemtrup et al., 2006; Irwin, 2009).

In our earlier work, we described the clinical course of babesiosis in dogs coming from areas of eastern Poland and disorders regarding selected haematological and biochemical parameters of the serum of dogs suffering from this disease (Adaszek et al., 2009).

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The clinical picture of infection with *B. canis* is diverse, ranging from hyperacute to acute and finally chronic disease (Adaszek et al., 2009). The main cause of death in the course of babesiosis is anoxia and shock (Bourdoiseau, 2006). According to our own observations, the death of dogs in which the disease was diagnosed and treatment was started was more frequently caused by complications associated with renal and hepatic insufficiency accompanied by elevated levels of serum urea and bilirubin (Adaszek et al., 2009).

Cases of cerebral babesiosis in dogs have been reported in increasing numbers recently (Adaszek et al., 2012; Daste et al., 2013; Leisewitz et al., 2019). The aetiology of this form of the disease has not been fully clarified (Keller et al., 2004). These complications may be attributed to disseminated intravascular coagulation, haemorrhage (Van de Maele et al., 2008) and hypoxia secondary to erythrocyte aggregation in capillaries, and inflammation (Schetters and Eling, 1999). Reperfusion injury may play a role in the pathogenesis of cerebral babesiosis (Vial and Gorenflot, 2006).

So far, no studies have been conducted to determine the serum concentration of manganese in dogs with babesiosis. Manganese (Mn) is neurotoxic when present in excessive concentrations (Racette et al., 2017). The mechanism of neurotoxicity appears to be multifactorial. The dysfunction of astrocytes occurs due to their high-affinity transport system, accumulating high intracellular concentrations of Mn (Yin et al., 2008). Manganese also causes microglial activation by the induction of cytokines and reactive oxygen species (Dodd and Filipov, 2011), and it also induces the disruption of several neurotransmitters and mitochondrial dysfunction, culminating in neuronal apoptosis (Smith et al., 2017).

There have been reports of dogs with anaemia with elevated serum concentrations of Mn, which may be a factor inducing neurological signs (Ferreira et al., 2017; Vitale et al., 2019). Since in the course of canine babesiosis anaemia is recorded in almost every case of the disease, the aim of this study was to demonstrate a link between the occurrence of signs of brain involvement in dogs with babesiosis and the concentration of Mn in their serum.

MATERIALS AND METHODS

Animals used in the study

Seven dogs with early babesiosis (Group 1), seven dogs with cerebral babesiosis (Group 2) and seven healthy dogs (Group 3) were studied prospectively between March and November 2019. All seven dogs with early babesiosis showed only signs of apathy. The dogs in Group 2 showed neurological signs. All animals of both Groups 1 and 2 were positive for the *B. canis* parasite in a thin stained blood smear and by polymerase chain reaction (PCR). The seven healthy dogs in Group 3 were admitted to our clinic for a routine check-up of their health status. They did not show any clinical abnormalities. In all animals, haematological and biochemical blood tests were performed.

Haematological and biochemical analyses

Venous blood was taken from the cephalic vein into tubes containing ethylenediaminetetraacetic acid (EDTA) for haematological and PCR evaluation, and into a plain tube for biochemical evaluation. The biochemical parameters measured in the serum were aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), total bilirubin, creatinine, urea, manganese (Mn), magnesium (Mg), sodium (Na), chloride (Cl), potassium (K) and calcium (Ca). Thin blood smears were stained by the Giemsa method and examined for parasites. Na, Cl, K and Ca were measured in the serum samples and determined in an automated analyser (BM ISE, BioMaxima, Poland) within 10 min of collection. The analyses of Mn and Mg levels were carried out using an inductively coupled plasma–optical emission spectrometry (ICP-OES Thermo iCAP 6000 series) at the Trace Element Analysis Laboratory of the Biophysics Department of Cerrahpasa Medical Faculty, Istanbul University-Cerrahpasa, Turkey. Each measurement was performed three times and averages were used for the analysis. The parameters of the ICP-OES device used for the determination of Mg and Mn are presented in Table 1. In the study, the appropriate wavelengths of Mg and Mn elements (285.213 nm and 257.610 nm, respectively) were used for analysis by the ICP-OES device.

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 amplify a 559-bp region of the 18S rRNA gene of *B. canis* (Adaszek and Winiarczyk, 2008; Łyp et al., 2015).

The real-time PCR reaction for all the isolated DNA samples was carried out using the Corbett apparatus. The real-time PCR with the SYBR Green 1 dye was carried out in thin-walled test tubes with a capacity of 100 µL. A DyNAmo HS SYBR Green qPCR Kit (Finnzymes) was used to conduct a 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 polymerase Tbr

Table 1. Parameters of the inductively coupled plasma–optical emission spectrometry (ICP-OES) device used for the determination of magnesium (Mg) and manganese (Mn) in blood serum samples

Parameters	Assigned value
Plasma gas flow rate	15 L/min
Argon carrier flow rate	0.5 L/min
Sample flow rate	1.51 L/min
Speed of peristaltic pump	100 rpm

(*Thermus brockianus*), buffer for the polymerase Tbr, deoxynucleotide triphosphate (dNTP), $MgCl_2$ and the intercalating SYBR Green 1 dye.

The optimised real-time PCR reaction included 50 subsequent cycles, each of them consisting of three stages: denaturation at 92 °C for 60 s, annealing at 52 °C for 60 s, and extension at 72 °C for 90 s (Adaszek and Winiarczyk, 2010).

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, Polish Academy of Sciences, Warsaw, Poland. DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison, USA), and ClustalV alignments 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 Mn concentrations as well as in other haematological and biochemical parameters between groups. Changes were considered statistically significant at $P < 0.05$. The Statistica 10.0 PL software was used for the calculations.

RESULTS

Results of the clinical, haematological and biochemical examinations

All dogs in Group 1 were apathic ($n = 7$; 100%). Dogs in Group 2 were anaemic ($n = 7$; 100%), and they showed the clinical signs of ataxia, paresis, seizures and altered consciousness ($n = 7$; 100%), nystagmus ($n = 3$; 43%) and opisthotonus ($n = 2$; 29%). Other complications recorded in animals in Group 2 were apathy ($n = 7$; 100%), brown colour of the urine ($n = 5$; 71%), icterus ($n = 3$; 43%) and diarrhoea ($n = 2$; 29%). The animals in Groups 1 and 2 were injected subcutaneously with imidocarb dipropionate solution (Imizol®, Schering Plough Animal Health). Four out of seven dogs (57%) in Group 2 died. None of the dogs from the control group (Group 3) showed any clinical signs of the disease. They were clinically healthy during the whole period of the study. The results of haematology and biochemistry are shown in Tables 2, 3 and 4.

The results of haematology showed a drop in haematocrit below 37% (the lower limit of the normal range) in two dogs in Group 1, all 7 dogs in Group 2 and no dogs in the control group. A decrease of red blood cell (RBC) count below 5.5×10^{12} (the lower limit of the normal range) was noted in one dog with early babesiosis, 7 dogs with neurological signs and no dogs in the control group. Leukopenia [white blood cell (WBC) count $<6 \times 10^9$] occurred in two patients in Group 1 (29%), three patients in Group 2 (43%) and no patients in the control group, while thrombocytopenia [platelet (PLT) count $<200 \times 10^9$] was reported in all dogs in Groups 1 and 2 and no dogs in the control group (Table 2).

Serum biochemical tests showed an increase in AST activity above the upper limit of the normal range (>37 IU/L) in three dogs in Group 1 (43%), 6 dogs in Group 2 (86%) and no dogs in the control group. An increase in ALT activity above the upper limit of the normal range (>50 IU/L) was recorded in two dogs in Group 1 (29%), 6 dogs in Group 2

Table 2. Results of haematological examinations of dogs from Groups 1, 2 and 3

Group	No.	WBC ($\times 10^9$)	RBC ($\times 10^{12}$)	Ht (%)	PLT ($\times 10^9$)
1	001	6.8	6.76	38.7	43
	002	5.0	7.29	45.0	28
	003	6.9	6.20	38.4	24
	004	4.3	6.54	40.5	27
	005	6.2	5.39	32.0	30
	006	7.8	5.98	34.4	65
	007	10.3	6.40	39.2	35
2	008	6.8	3.78	24.8	19
	009	6.1	3.97	25.2	31
	010	9.3	4.3	25.6	39
	011	4.1	4.73	29.7	16
	012	7.7	5.08	32.4	48
	013	5.6	4.75	29.7	28
	014	4.8	5.07	31.7	29
3	015	10.4	6.4	42.1	231
	016	9.8	6.54	39.9	379
	017	8.9	6.86	44.4	224
	018	8.8	7.12	49.6	443
	019	6.8	6.54	39.9	379
	020	9.9	6.16	42.1	220
	021	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 3. Results of serum biochemical examination of dogs from Groups 1, 2 and 3

Group	No.	ALT (IU)	AST (IU)	BIL T (mg/dL)	AP (IU)	Urea (mg/dL)	Creatinine (mg/dL)
1	001	34	35	0.58	112	43.1	1.32
	002	30	22	0.48	131	39.5	1.8
	003	45	31	0.55	98	42.1	1.16
	004	62	44	0.42	147	44.0	1.61
	005	70	42	0.40	115	39.2	1.52
	006	48	26	0.66	124	40.6	1.33
	007	45	39	0.54	130	43.7	1.86
2	008	41	32	0.98	132	45.1	1.42
	009	74	73	1.52	77	51.6	1.86
	010	126	49	0.73	115	35.0	0.67
	011	74	61	0.86	124	49.5	1.59
	012	182	148	0.88	95	34.5	0.76
	013	107	75	0.73	134	47.9	1.84
	014	87	101	0.79	98	40.2	0.98
3	015	44	19	0.42	110	43.7	1.14
	016	26	27	0.58	147	42.7	1.14
	017	30	33	0.47	145	35.1	1.52
	018	33	35	0.22	142	31.6	1.44
	019	48	22	0.59	108	42.9	1.48
	020	34	30	0.44	129	43.8	0.91
	021	29	19	0.34	114	32.5	0.61
	Range	3–50	1–37	≤0.60	20–155	20–45	1.00–1.70

ALT = alanine aminotransferase; AST = aspartate aminotransferase; AP = alkaline phosphatase.

Table 4. Macroelement and trace element levels in the serum of dogs used in the study

Group	No.	Na (mmol/L, mEq/L)	K (mmol/L, mEq/L	Cl (mmol/L, mEq/L	Ca (mmol/L, mEq/L)	Mn (μg/mL)	Mg (mg/L)
1	001	140.0	5.3	113.7	2.6	0.007	20.34
	002	145.6	5.1	115,2	2.1	0.004	24.41
	003	145.1	4.2	114.8	3.0	0.004	24.52
	004	144.8	3.9	115,6	2.2	0.005	17.76
	005	148.0	4.6	118.9	2.2	0.005	22.51
	006	147.4	4.4	116.1	2.4	0.008	17.29
	007	147.3	4.8	118.2	2.5	0.006	17.40
2	008	148.0	4.1	114.0	2.3	0.014	18.44
	009	141.1	5.7	118.2	2.3	0.013	25.16
	010	145.3	5.9	115.0	2.7	0.016	20.17
	011	145.7	4.7	115.6	3.0	0.014	19.12
	012	137.6	5.6	112.6	2.6	0.014	17.80
	013	149.1	5.0	112.9	2.1	0.015	21.54
	014	144.6	6.0	113.8	2.9	0.017	22.61
3	015	145.0	3.9	115.3	2.6	0.007	23.14
	016	147.2	4.6	114.3	2.4	0.004	18.14
	017	144.2	4.3	118.4	2.5	0.010	17.88
	018	148.6	4.4	117.4	2.4	0.009	23.20
	019	148.2	5.1	117.8	2.4	0.009	21.85
	020	145.9	3.9	116.1	2.7	0.008	19.10
	021	144.8	5.1	116.5	2.2	0.006	20.55
	Range	144.2–149.8	3.9–5.2	114.1–118.6	2.1–3.0	0.004–0.012	17–29

Na = sodium; K = potassium; Cl = chloride; Ca = calcium; Mn = manganese; Mg = magnesium.

(86%) and no dogs in the control group. In turn, an increase in total bilirubin above the upper limit of the normal range (<0.6 mg/dL) was recorded in one dog in Group 1 (14%), 7 dogs in Group 2 (100%) and no dogs in the control group. The activity of alkaline phosphatase (AP) was maintained within the physiological range (>155 IU/L) in all dogs in the study and control groups. An increase in urea concentration above the upper limit of the normal range (>45 mg/dL) was noted in only 4 patients in Group 2 (57%). Elevated creatinine levels above the upper limit of the normal range (>1.7 mg/dL) were found in two dogs in Group 1 (29%), two dogs in Group 2 (29%) and no dogs in the control group (Table 3).

Potassium concentration was above normal in one dog in Group 1 (14%), 3 dogs in Group 2 (43%), while the dogs in the control group showed normal concentration. Hypochloraemia was present in one out of seven dogs in Group 1 (14%) and in 4 out of 7 dogs in Group 2 (57%). Hyperchloraemia was identified in one dog in Group 1 (14%). The remaining animals included in the study showed normal levels of chloride. Serum sodium concentration was low in one dog in Group 1 (14%), and two dogs in Group 2 (29%), while the remaining dogs showed normal Na concentration. Calcium concentration was normal in all 21 dogs (100%).

Serum Mg concentrations in all dogs, including dogs with early babesiosis (Group 1), neurological babesiosis (Group 2) and those in the control group (Group 3), were within normal limits, while Mn concentrations were elevated (average: 0.015 µg/mL) in all dogs in Group 2. In dogs in Groups 1 (average: 0.006 µg/mL) and 3 (average: 0.008 µg/mL), the serum concentration of this element was within the normal range (Table 4).

Results of the molecular examinations

Babesia canis DNA was found in blood samples taken from all 14 ill dogs examined by real-time PCR. The products were visualised by agarose gel electrophoresis. Their size compared with the mass standard was about 559 bp. The Ct values read from the amplification curve fluctuated around 17 cycles for all the samples examined. The melting temperature (Tm) ranged between 78 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. Three out of four dogs that died in the course of the disease were infected with *B. canis* 18S RNA-B strain, and one with *B. canis* 18S RNA-A strain.

Statistical results

Among the parameters studied, statistically significant differences were noted only in serum Mn concentration between dogs of Group 2 and dogs of Groups 1 and 3 ($P = 0.002$ and $P = 0.029$, respectively). The average concentration of Mn in dogs in Group 2 was 0.026 µg/mL, whereas in Groups 1 and 3 it reached 0.0055 and 0.0076 µg/mL, respectively.

DISCUSSION

Cerebral babesiosis (CB) has been described or suspected in infections with *Babesia bovis* and *Babesia bigemina* in cattle (De Vos et al., 1976), *Babesia caballi* in horses (Malherbe, 1956) and *B. canis* in dogs (Máthé et al., 2006; Van de Maele et al., 2008; Adaszek et al., 2012). This form of infection develops only in a small proportion of infected animals. Exact figures are not available, but less than 0.1% of 1,200 African dogs assessed for rabies over a period of three years (that had died with cerebral pathology) showed a massive accumulation of infected erythrocytes in the brain, which is suggestive of low incidence (Okoh, 1978). CB can result from either sludging of parasitised erythrocytes in small vessels of the brain or metabolic derangement. Tissue hypoxia can lead to neurological signs (Boozer and Macintire, 2003). Immune complex deposition in blood vessels of the brain and vasculitis in the course of the disease (Shaw et al., 2001), as well as disseminated intravascular coagulation (DIC), can also lead to the development of CB (Máthé et al., 2006). Cerebral malaria may result from nitric oxide-induced changes of neurotransmission. A similar pathogenesis could also hold true for canine babesiosis (Schetters and Eling, 1999; Jacobson, 2006). CB is usually associated with high mortality (Boozer and Macintire, 2003). Clinical signs such as seizures and altered consciousness, similar to those described in this study, have been reported (Boozer and Macintire, 2003; Adaszek et al., 2012).

No studies have been conducted so far to demonstrate a correlation between blood Mn concentration in dogs with babesiosis and the occurrence of neurological disorders in the course of the disease.

The main finding of this study was that serum Mn concentrations were statistically significantly higher in dogs with neurological babesiosis compared with dogs with early babesiosis and healthy dogs ($P = 0.002$ and $P = 0.029$).

No other factors were shown to cause neurological signs in this study. The results of a cranial CT scan in dogs with neurological disorders as well as the results of the autopsy and histopathological examination of the brain of two dead dogs (data not shown) did not reveal any changes similar to those previously described in dogs with cerebral babesiosis (Van de Maele et al., 2008; Adaszek et al., 2012). Furthermore, the differences in electrolyte levels between the different study groups were not significant enough to allow the assumption that they might have been a trigger for neurological signs in dogs in Group 2. Neurological signs in dogs may be the consequence of a reduced serum Mg concentration (Cronin et al., 1982), but in all animals used in the study, serum Mg was within the physiological limits. On this basis, it was assumed that an increased concentration of Mn could have had an impact on the occurrence of neurological disorders.

Increased serum concentrations of Mn in the second group of dogs may be associated with the development of anaemia. In humans with anaemia, increased intestinal iron absorption also results in the increased absorption of other trace elements, including Mn (Meltzer et al., 2010). It can be assumed that the situation is similar in dogs.

In contrast to this, in anaemic cattle infected with *B. bigemina* decreased serum levels of copper, zinc, manganese and selenium along with increased levels of iron were observed. It can be assumed that the decreased levels of trace elements (including Mn) represent their co-ordinated antioxidant role along with antioxidant enzyme activities during the infection (Esmaeilnejad et al., 2020).

The presence of clinical Mn neurotoxicity with concurrent anaemia, and without Mn exposure, has been suggested in developing mice and confirmed in one human child (Kwik-Uribe et al., 1999; Brna et al., 2011).

It is worth noting that liver function was impaired in dogs in Group 2 compared to Groups 1 and 3, which was

supported by increased AST and ALT activities. Dogs with hepatic dysfunction and portosystemic shunting have altered Mn homeostasis, leading to Mn accumulation (Kilpatrick et al., 2014). In particular, the liver plays a pivotal role in Mn metabolism as the majority of gastrointestinally absorbed Mn is removed by the liver and excreted into the bile so that only approximately 2% of absorbed Mn reaches the systemic circulation (Aschner and Aschner, 2005).

The main pathological consequence of increased Mn concentrations in hepatic disorders is considered to be a neurological dysfunction. The exact mechanism by which Mn causes encephalopathy is unclear, although there are both *in vitro* and *in vivo* studies which suggest that many of the effects are centred on the astrocyte, a key cell in the development of hepatic encephalopathy (Prakash and Mullen, 2010).

The finding that Mn concentrations are increased in some dogs with CB may be therapeutically relevant since chelation treatment with para-aminobenzoic acid and disodium edetate has been used successfully in human cases of Mn toxicity (Herrero Hernandez et al., 2006). This has led to the suggestion that it may be of use in controlling Mn and thus hepatic encephalopathy in humans and dogs (Torisu et al., 2008; Tuschl et al., 2008). In support of this, Park et al. (2008) used trientine chelation therapy in one human case of chronic hepatic disease with acquired portosystemic shunting and hepatic encephalopathy. Following treatment, there was a decrease in blood Mn concentrations and a reduction in hyperintensity demonstrated by magnetic resonance imaging in the central nervous system (CNS), which was thought to be consistent with a reduction in Mn deposition in the CNS. These findings correlated with a reported marked improvement in clinical signs.

Based on the data obtained in this study, it can be concluded that canine CB due to infection with *B. canis* may be associated with anaemia, liver failure, and increased serum Mn concentrations. Given the well-established neurotoxic effects of Mn in humans, experimental rodents and primates, additional studies on the role of Mn in the pathogenesis of CB in dogs are warranted.

We could hypothesise that babesiosis was the cause of the high Mn concentration, but in that case hypermanganesaemia should have been detected in all dogs with babesiosis from Groups 1 and 2, and not only in animals of Group 2.

Further molecular and biochemical studies are required to reach a better understanding of the pathogenesis of *B. canis* infection, especially of the atypical form of the disease. In addition, efficient biomarkers for the early diagnosis of this parasitic disease should be sought and investigated.

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