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PROTOZOOLOGY - ORIGINAL PAPER

Differential acute-phase protein responses in dogs seropositive or seronegative for *Neospora caninum*

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Abstract

Neospora caninum is one of the most prevalent Apicomplexa parasites that causes abortion in cattle, as it infects dogs as its definitive host, causing subclinical disease or active neosporosis, marked by meningoencephalitis, and myopathies with muscle and neuromuscular signs of disease. This study aimed to evaluate the acute phase protein response in dogs seropositive and seronegative for *N. caninum*. Serum samples of 72 dogs were tested by an immunofluorescence antibody test using *N. caninum* NC-1 strain, and the study population was divided into four groups: symptomatic — muscular and/or neuromuscular signs — and seropositive ($n=16$); symptomatic and seronegative ($n=9$); asymptomatic and seropositive ($n=34$); and asymptomatic and seronegative ($n=13$). C-reactive protein (CRP) was measured via immunoturbidimetric assay and serum haptoglobin (Hp) via hemoglobin-binding capacity assay. In the symptomatic groups, seropositive dogs had higher levels of Hp, but not CRP, while seronegative dogs had higher CRP levels. There was no difference in CRP concentration in asymptomatic dogs. Dogs with neuromuscular signs had higher concentrations for Hp in the group seropositive. Hp concentration did not differ between dogs seropositive and seronegative dogs for each group. Serum Hp and CRP could not sufficiently alone flag subclinical infections. Measurement of CRP and Hp concentrations could be clinically valuable to the diagnosis of neurological diseases, and their relative change may indicate the stage of the infection, although their sole use is not able to support the diagnosis of canine neosporosis. Further studies are encouraged to evaluate the specific dynamics of acute phase proteins in canine neosporosis.

Keywords Neosporosis · CRP · Haptoglobin · Immunofluorescence antibody test · Neurological · Muscular

Introduction

Neospora caninum is an apicomplexan parasite and the causative agent of neosporosis, a major cause of abortion in cattle and neuromuscular disease in dogs (Donahoe et al., 2015). Canine neosporosis was first recognized in Norway (Bjerkås et al., 1984) and has emerged as a serious disease worldwide (Dubey and Lindsay, 1996; Nazir et al., 2014), as it has been reported in Italy (Cringoli et al., 2002; Pasquali et al., 1998), in UK (Coelho et al. 2019; Knowler and Wheeler, 1995) in South Africa (Jardine & Dubey, 1992), in the USA (Ruehlmann et al., 1995), and Brazil (Cerqueira-Cézar et al., 2017; Fridlund-Plugge et al. 2008).

Clinical neosporosis has been also reported in sheep, goats, deer, rhinoceros, and horses, and antibodies to *N. caninum* have been found in the sera of water buffaloes, foxes, coyotes, camels, and felids (Buxton et al. 2002). There are a great number of species that serve as intermediate hosts for *N. caninum*, but wild canids and domestic dogs are the only known definitive hosts (Dubey, 2003). Canine neosporosis is the most common cause of infectious inflammatory myopathies in dogs around the world (Podell, 2002) and is an important cause of meningoencephalitis, polymyositis, and polyradiculoneuritis (Gaitero et al., 2006; Shelton, 2007; Garosi et al., 2010).

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The acute phase response (APR) is a part of the complex set of systemic reactions of the body's early defense, seen shortly after stimuli including stress, inflammation, and infection. Acute phase proteins (APP) are components of the APR that act to reestablish homeostasis and promote healing (Ceron et al., 2005; Eckersall and Bell, 2010). Although considered being non-specific innate immune components, the circulating concentrations of the APP are related to the severity of the disorder and extent of damage in the affected animal, which can provide diagnostic and prognostic information, but there are differences in response between the individual APP in different diseases (Ceron et al. 2014; Murata et al., 2004).

C-reactive protein (CRP) has been demonstrated in dogs (Caspi et al., 1984) and is considered one of the major APP in dogs (Caspi et al., 1987; Conner et al., 1988). The concentration of CRP in the blood can increase over 100 times within 24 h of the initiation of damage caused by infection, inflammation, or trauma (Eckersall et al., 1999). Haptoglobin (Hp) is a transport protein that binds hemoglobin. It shows a moderate increase during inflammation in dogs, increasing 2–5 times (Paltrinieri, 2007). As an APP of moderate response in dogs, it peaks after 2 to 3 days and decreases more slowly than CRP (Eckersall and Bell, 2010). The APP responds to some parasite infections in dogs, notably babesiosis (Kuleš et al., 2014) and Leishmaniosis (Ceron et al., 2018), but there have been no reports on the APP response in dogs infected with *N. caninum*. Overall, APP can respond differently and not proportionally to diverse inflammatory stimulus (McGrotty et al. 2003; Ceron et al., 2018).

We hypothesized that dogs infected with *N. caninum* have pathophysiological changes activating the acute phase response and an increase the concentration of acute phase proteins in serum; thus, this study aimed to explore the serum concentration of Hp and CRP in dogs seropositive and seronegative for *N. caninum* presenting neuromuscular signs or in subclinical infections without neuromuscular signs.

Methods

Animals

The study population comprised of 72 client-owned mixed breed dogs (38 males and 34 females), admitted to the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine of Federal University of Paraná (HV-UFPR), Brazil. Dogs included in the study were dogs with neuromuscular signs such as seizures, tremors, hyperesthesia, progressive paralysis, and stiffness of the limbs or dogs being admitted to the hospital for medical elective purposes such as neutering, vaccines, or routine checkups, and with no abnormal findings on physical examination or any other considerable alterations in hemogram and biochemical examination of serum (control group). All dogs were tested by indirect fluorescent antibody test (IFAT) and then divided into four groups: (1) dogs with muscular and/or neuromuscular signs and seropositive for *N. caninum* ($n=16$); (2) dogs with muscular and/or neuromuscular signs and seronegative for *N. caninum* ($n=9$); (3) dogs seropositive for *N. caninum* with no neuromuscular signs ($n=34$); and (4) healthy dogs and seronegative for *N. caninum* ($n=13$). The study was approved by the Animal Use Ethics Committee on the Agricultural Sciences Campus of the Federal University of Paraná (Protocol number 065/2016). All institutional and national guidelines for the care and use of animals were followed.

Laboratory testing

Indirect fluorescent antibody test

Blood samples were obtained by jugular vein puncture, placed in plain tubes and were allowed to clot at room temperature, centrifuged ($1500\times g$ for 5 min) and the harvested sera were stored in Eppendorf microtubes at − 80 °C until further analysis. IFAT was performed at the Laboratory of Veterinary Clinical Pathology of HV-UFPR. Slides were prepared with tachyzoites of *N. caninum* NC-1 strain, from culture in Vero cells at the same laboratory. Dog sera were diluted at 1:50 in PBS, pipetted into each well of the antigen-containing slides, and incubated for 30 min at 37 °C in a humidified chamber. Slides were washed in PBS (5 min), and subsequently secondary antibody was added (diluted 1:100, IgG anti-dog with fluorescein isothiocyanate, Abcam, UK) and incubated for 30 min at 37 °C in a humidified chamber. Following two washes (PBS for 10 min; distilled water for 5 min), slides were mounted (cover glass and glycerin 90%), and assessed using an Olympus BX60 epifluorescent microscope immediately after preparation. Only samples that exhibited fluorescence of the entire parasitic surface were considered to be positive. A positive and a negative dog serum sample by PCR were included in all slides as an assay control. Positive samples were also examined at dilutions of 1:100, 1:200, 1:400, and 1:600 until final titers were reached. Samples were also tested for *Sarcocystis neurona* (SN37R strain) and *Toxoplasma gondii* (RH strain) using the same method, and positive samples (1:50) were removed from the study (not reported as study population).

Acute phase proteins

CRP was measured with a validated canine-specific immunoturbidimetric assay (Piñeiro et al., 2018) (Gentian CRP, Gentian AS, Moss, Norway). Serum Hp was measured via hemoglobin-binding capacity assay, based on the method of Eckersall et al. (1999) method and modified as described

in Brady et al. (2018). Both assays were performed on Architect c4000 automated biochemistry analyzer (Abbott Diagnostics, IL, USA) respectively in a single run. The biochemical determinations of APP were carried out at the Laboratory of Internal Diseases Clinic of Faculty of Veterinary Medicine, University of Zagreb, Croatia.

Statistical analysis

All statistics were performed using EZR 1.37 (Easy R, Saitama Medical Center, Jichi Medical University, Kanda, 2013), which is a graphical interface for R commander (The R Foundation for Statistical Computing, Vienna, Austria, version 3.4.4). Data are reported as median and range. The non-parametrical Mann–Whitney *U* test was used to compare results obtained for dogs seronegative and seropositive to *N. caninum* and also for comparison between dogs with or without neuromuscular signs. A Kruskall–Wallis analysis of variance was used to compare the results between all four groups. Statistical significance was set at $P < 0.05$ for all analyses.

Results

CRP concentrations were higher in seronegative dogs with neuromuscular signs (group 2) than in seropositive dogs with neuromuscular signs (group 1) (comparison between groups 1 and 2; $W = 112$, $p = 0.023$; Table 1 and Fig. 1). Median CRP concentrations did not differ between clinically healthy dogs seropositive for *N. caninum* (group 3) and clinically healthy dogs seronegative for *N. caninum* (group 4) ($W = 204.5$, $p = 0.703$). CRP concentrations were low in *Neospora* positive animals in both clinically healthy dogs (group 1) and dogs with neuromuscular signs (group 3) (groups 1 and 3, $W = 251.5$, $p = 0.6774$).

Dogs with neuromuscular signs had higher concentrations for Hp in groups seropositive for *N. caninum* (groups 1 and 3) than seronegative for *N. caninum* (group 2 and 4) (comparison between groups 1 and 3 = 114, *p* value = 0.001; comparison between groups 2 and 4: W = 23, *p* value = 0.017; Table 1 and Fig. 2). There was no significant difference in Hp concentrations in dogs seropositive for *N. caninum* with neuromuscular signs (group 1) and seronegative animals with neuromuscular signs (group 2) (comparison between groups 1 and 2; $W = 77$, $p = 0.803$), nor in clinically healthy dogs seropositive for *N. caninum* (group 4) and clinically healthy seronegative animals (comparison between groups 3 and 4; $W = 219$, $p = 0.971$).

There were more male dogs seropositive for *N. caninum* than females ($p = 0.0227$), but no difference between the number of males and females in dogs with clinical signs ($p = 0.46$). Considering all groups, male dogs had higher Hp concentration ($W = 825.5$, $p = 0.046$) than females, but for CRP concentration there was no difference ($W = 717.5$, $p = 0.4232$) between males and females (Table 2).

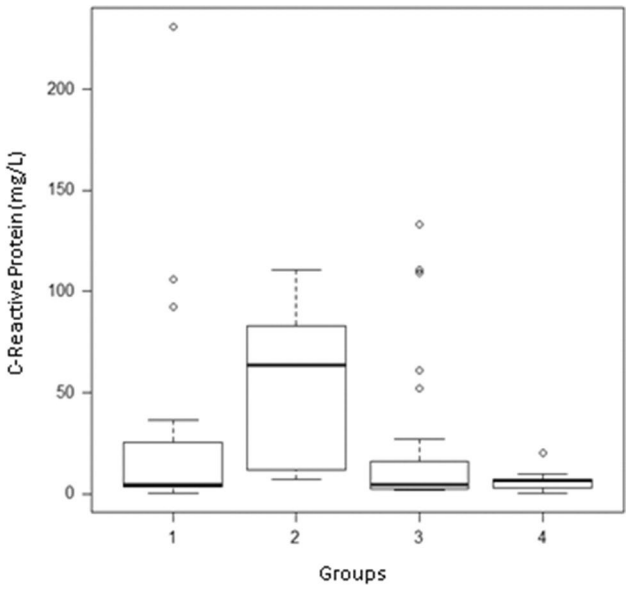

Fig. 1 Box plot of CRP serum concentration in different groups. CRP concentrations in dogs seropositive and seronegative for *N. caninum*, displaying or not neuromuscular signs (groups 1, 2, 3, and 4 as shown in Table 1). The lower (Q1) and upper (Q3) quartile, representing observations outside the 9–91 percentile range. The diagram also shows the median and mean observation for a particular group. Data falling outside the Q1–Q3 range are plotted as outliers of the data. (Kruskal–Wallis $X^2 = 11.046$; $p = 0.011$)

Table 1 CRP and haptoglobin concentrations in different groups

Groups	Number of dogs (*n*)	CRP (mg/L) median [range]	Haptoglobin (g/L) median [range]
(1) Dogs with neuromuscular signs and seropositive for *N. caninum*	16	4.7^{x} [0.5–230.9]	2.26^{a} [0.86–7.80]
(2) Dogs with neuromuscular signs and seronegative for *N. caninum*	9	63.7^{y} [7.1–110.8]	2.79^{a} [0.93–5.94]
(3) Healthy dogs and seropositive for *N. caninum*	34	$4.6^{x,z}$ [1.9–132.9]	1.03^{b} [0.23–5.75]
(4) Healthy dogs and seronegative for *N. caninum*	13	6.8^{z} [0.5–20.4)	0.93^{b} [0.31–3.57]

Results of CRP and haptoglobin concentrations in dogs seropositive and seronegative for *N. caninum*, displaying or not clinical signs. The same superscripts indicate that there is no significant difference in the haptoglobin (a, b) or CRP (x, y, z) results

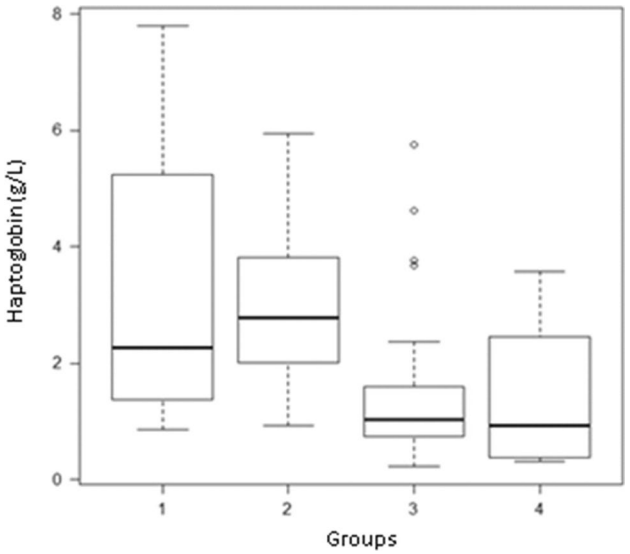

Fig. 2 Box plot of haptoglobin serum concentration in different groups. Haptoglobin concentrations in dogs seropositive and seronegative for *N. caninum*, displaying or not neuromuscular signs (groups 1,2, 3, and 4 as shown in full in Table 1). The lower (Q1) and upper (Q3) quartile, representing observations outside the 9–91 percentile range. The diagram also shows the median and mean observation for a particular group. Data falling outside the Q1–Q3 range are plotted as outliers of the data. (Kruskal–Wallis $X^2 = 16.723$; p value < 0.001)

Titers in seropositive dogs ranged from 1:50 to 1:200. These titers were grouped in 3 categories for statistical analysis: (i) seronegative, (ii) seropositive (1:50 to 1:200), and (iii) indicative of active neosporosis (> 1:200). No association between titers and concentration of both APP were detected (CRP: Kruskall–Wallis $X^2 = 1.7179$, $p = 0.423$; Hp: Kruskall–Wallis $X^2 = 0.64468$, $p = 0.72$).

The correlation between CRP concentration and Hp concentration is shown in Fig. 3. The concentration of both APP was dissociated in many cases, and by using Spearman's rank correlation coefficient, only a weak correlation was observed ($r = 0.44$, $p < 0.001$).

Discussion

Clinical neosporosis is marked by neurological and muscular signs, including forelimb atrophy, gradual muscular rigidity, ataxia, seizures, nystagmus, hypermetria, and cervical hyperesthesia (Dubey, 2003; Sykes 2014), and can be challenging to differentiate from other neurological diseases (Jacques et al., 2002). Neosporosis diagnosis is usually reached through the association of clinical history, epidemiologic factors, and serological examinations (Dubey et al. 2007), being IFAT the gold standard (Björkman and Uggla, 1999; Silva and Machado 2016). APP are mainly used as broad markers of inflammation and infection, aiding the identification of many diseases (Murata et al., 2004). In this study, we explored the CRP and Hp responses in seropositive and seronegative dogs with different clinical backgrounds, aiming to evaluate the potential diagnostic support of these APP is canine neosporosis.

The decreased concentration of serum CRP in the seropositive dogs with clinical signs may be useful to distinguish between neuroinflammatory diseases from other neuropathies. Canine idiopathic polyarthritis, which has similar signs to *N. caninum*, such as fever, lameness, and inability to walk, has been associated with higher expression of CRP (Ohno et al., 2006). Similarly, dogs with steroid responsive meningitis arteritis also show signs as paralysis, leg weakness, and stiff gait, and have markedly elevated CRP levels in serum and CSF (Bathen-Noethen et al., 2008; Kordass et al., 2016; Lowrie et al., 2009). A contrasting response is found in meningoencephalitis, a typical characteristic lesion for *N. caninum* (Galgut et al. 2010; Donahoe et al., 2015), which has been associated with low CRP concentration in dogs (Nakamura et al. 2008). The seropositive dogs with elevated CRP may be responding to the active multiplication of the tachyzoites, which can be inducing the destruction of neuronal cells (Silva and Machado, 2016, Peters et al. 2001). These are at the stage when the infection is going through an active phase and pro-inflammatory cytokines are being secreted and stimulating the acute phase reaction and production of CRP. On seroconversion, with the activation of the acquired immune system to produce antibodies to *N. caninum*, the acute phase of the innate immune response and

Table 2 CRP and haptoglobin concentrations in males and females

	Number of dogs (*n*)	Number of seropositive	CRP (mg/L) median [range]	Haptoglobin (g/L) median [range]
Male	38	31	6.54 [0.5–230.9]	3.18 [0.23–7.72]
Female	34	19	4.78 [0.5–132.9]	1.95 [0.31–7.80]

Results of CRP and haptoglobin concentrations in female and male dogs, considering the groups altogether

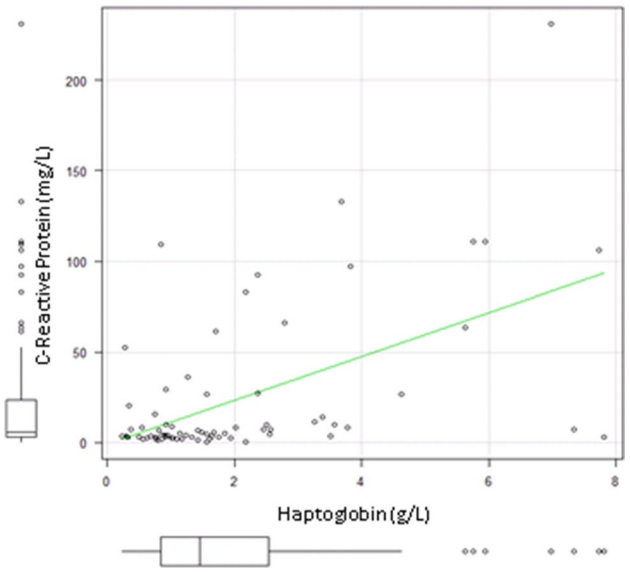

Fig. 3 CRP vs haptoglobin concentrations. Scatterplot of correlation of CRP concentration and haptoglobin concentration in 72 dogs (all groups). Spearman's rank correlation coefficient was $r = 0.44$ ($p < .001$)

cytokine production is dampened down and no longer causing an increase in CRP. However, studies including the long-term repeated analysis of CPR through the infection course are necessary to evaluate the dynamics of this APP in neosporosis, and CRP alone is not sufficient to rule out other diseases.

Serum Hp concentrations showed an increase in dogs with clinical signs, regardless of seropositivity. Neosporosis can also induce a variety of lesions depending on the parasitized cells, other than neuromuscular cells, causing myocarditis, polymyositis, pancreatitis, and interstitial pneumonia with pulmonary edema and alveolitis (Greig et al. 1995; Ordeix et al. 2002; Barber and Trees, 1996). Together with the stage of the infection, this could also explain the outliers with high levels of APP in seropositive dogs. Hp is a moderate APP in dogs and the rise in Hp concentration prior to seroconversion extends longer into the period when a seropositive reaction is present. In addition, serum Hp in dogs is known to respond to increased cortisol, and it may be that this steroid hormone is also increased due to the stress of the infection, while the presence of cortisol does not alter the production of CRP (Caldin et al., 2009). The two acute phase proteins have different profiles of response in dogs (Eckersall and Bell, 2010), which can also explain the weak correlation found between both APP measurements.

When comparing to other protozoan infections, the increases in the APP following infection with *N. caninum* are less than those found in babesiosis and Leishmaniosis (Kuleš et al., 2014; Ceron et al 2018) and is presumably due to differences in pathogenesis between the infections. *N. caninum* isolates have reportedly intra-specific variability which could lead to different virulence (Schock et al., 2001; Miller et al., 2002; Pérez-Zaballos et al., 2005). This diversity may be associated with the clinical presentation of the disease (Rojo-Montejo et al., 2009). An isolate with low virulence has been reported in southern Brazil (Locatelli-Dittrich et al., 2018) where samples from this study were collected, which could explain the lower pro-inflammatory response increases in APP found in this study compared to other parasite infections, and the lack of association between titers and concentration of both APP evaluated.

Dogs can also be sub-clinically infected, which allows the transmission between bitches to their fetuses (Donahoe et al., 2015). Neither CRP nor Hp was able to flag subclinical infections, although an increase in both APPs was reported in a few seropositive dogs in the healthy groups which could be possibly associated with response triggered by early infection of tachyzoites in different tissues, and differentiation to bradyzoites to form tissue cysts (Silva and Machado, 2016). APP response can help monitor animal health and could help identify infectious diseases in an early stage, including neosporosis, yet the lack of specific response and the inability to identify seropositive groups in the asymptomatic groups anticipate limited use of Hp supporting the diagnosis of canine neosporosis. Still, it could be that decreased serum concentration of CRP and increased Hp concentration in dogs with neuromuscular signs, and with no other clear cause of an acute phase reaction, might be a piece of valuable information to raise awareness to canine neosporosis and seek immunological confirmation or DNA detection.

Conclusion

In conclusion, seropositive dogs with neuromuscular signs presented higher levels of Hp, but not CRP. CRP or Hp was not able to identify subclinical infections. Measuring CRP concentrations may be clinically valuable to distinguish between neurological diseases, since chronically infected dogs for *N. caninum* maintain low levels of CRP after seroconversion, differently from other diseases such canine idiopathic polyarthritis or steroid responsive meningitis arteritis, which are marked with high levels of CRP. Further studies are encouraged to evaluate variation CRP levels with the progress of neosporosis and different clinical courses in dogs.

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Declarations

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study was approved by the Animal Use Ethics Committee on the Agricultural Sciences Campus of Federal University of Paraná (Protocol number 065/2016).

Competing interests The authors declare no competing interests.

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