



Short Communication

Horses Seropositive for *Neospora* spp.: Immunoglobulins, Cytokines, and C-Reactive Protein Levels

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ABSTRACT

Neosporosis is a disease known to cause reproductive problems in cattle. In horses, the disease is caused by the protozoa *Neospora caninum* and/or *Neospora hughesi*, but little is known about this infection in this species. Therefore, the aim of this study was to quantify the levels of immunoglobulins (Igs), cytokines, and acute phase protein in seropositive horses for *Neospora* spp. (n = 23) comparing with seronegative horses (n = 20). There was no significant difference ($P > .05$) between IgG levels; however, IgM levels were significantly higher in sera samples from positive animals ($P < .01$). Similarly, proinflammatory cytokines (tumor necrosis factor, interferon gamma, interleukins [IL-1, IL-4, and IL-6]) and C-reactive protein levels were also significantly higher ($P < .01$) in horses seropositive for *Neospora* spp. On the other hand, the IL-10 levels (anti-inflammatory cytokine) were lower in horses seropositive for neosporosis ($P < .01$). Therefore, we conclude that horses naturally infected by *Neospora* spp., even without clinical disease, are able to activate their immune systems to control the infection, which may lead to disease chronicity.

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1. Introduction

Neosporosis is a disease caused by an obligate intracellular protozoan that affects various domestic animals species such as cattle, dogs, sheep, goats, buffaloes, and horses [1–5], and among the most affected species is cattle. Infection with the protozoan parasite *Neospora caninum* is thought to be a major cause of reproductive failure in dairy cattle worldwide [6,7]. Cattle infected with the parasite are three to seven times more likely to abort compared with uninfected cattle [6]. The parasite may be transmitted to cattle through the ingestion of oocysts that are shed in the feces of acutely infected dogs (definitive host of *N. caninum*)

or by congenital infection via placenta [2,6,8]. In horses, the neosporosis can be caused by protozoa *N. caninum* and/or *Neospora hughesi*; however, the *N. caninum* is best known and studied [9]. Yet, the life cycle of *N. hughesi* has not been fully established and the definitive host is unknown, just like other possible intermediate hosts besides horses [10], beyond that there is no specific treatment for neosporosis in horses. Therefore, further studies are needed also to better understand the pathogenesis of this disease in horses.

According to the literature [11], *Neospora* spp. is related to reproductive disorders such as infertility, abortion, and neonatal mortality in horses and a cell-mediated immune response is mounted by the host to control infection by *N. caninum*. During an experimental *N. caninum* infection, there is a typical cellular response by T lymphocytes helper type 1 (Th1), characterized by high levels of interferon gamma (IFN- γ), and a humoral response mediated by

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immunoglobulin (Ig) G2 in cattle [12]. This inflammatory response of the host will determine the tachyzoites infection, that is, to determine the ability of parasite to penetrate and multiply in the host cells [13].

Studies in nonpregnant cattle and murine models of infection have shown the importance of T-helper 1-type immune responses involving proinflammatory cytokines, such as IFN- γ and interleukins (IL-12), in limiting intracellular multiplication of the parasite [6,7]. During pregnancy, some changes occur in the immune system allowing the mother to accept the fetus; and the crucial role of T-helper 2-type cytokines at the maternal–fetal interface in maintaining the pregnancy and regulating the potentially damaging effect of Th1 responses [6]. Unlike cattle, in horses naturally infected by *Neospora* spp., very little is known about the influence of the disease in immune response or vice versa. As a consequence, the aim of this study was to investigate Igs, cytokines, and acute phase proteins in serum samples from *Neospora* spp. seropositive horses.

2. Material and Methods

Serum samples stored at -20°C collected from horses naturally infected by *Neospora* spp. (seropositive) was used in the present study. A previous study was conducted in Santa Catarina state, Brazil, using immunofluorescence assay for *Toxoplasma gondii* and identified 23 positive animals with titers of 1:50 ($n = 12$), 1:100 ($n = 8$), and 1:200 ($n = 3$) [14]. The samples of noninfected horses used in this study were seronegative to *Neospora* spp. and *T. gondii*. Therefore, the stored sera from 20 of seronegative horses (control) and 23 of seropositive horses to *Neospora* spp. were used to evaluate Igs, cytokines, and C-reactive protein (CRP) levels.

Serum IgG and IgM levels were determined using immunonephelometry on the Behring Nephelometer BN II (Dade Behring) with reagents from Dade Behring and specific kits for IgG (Horse IgG(T) ELISA Quantitation Set; Bethyl Laboratories) and IgM (Horse IgM ELISA; Kamiya Biomedical Company, Seattle). Samples were analyzed according to Dati et al [15]. Briefly, all samples were diluted with specific diluents and measured after 10 minutes. Polystyrene wells were coated with a specific monoclonal antibody for each serum protein forming an agglutinate that disperses the light irradiated in the presence of the protein. The intensity of scattered light depends on the amount of protein concentration in the sample, and the results are compared with known standard curves [15].

Cytokine quantification (Tumor necrosis factor [TNF- α], IFN- γ , IL-1, IL-4, IL-6, and IL-10) was assessed by enzyme-linked immunosorbent assay using commercial Quantikine immunoassay kits (GSI Equine—Plasma/Serum DataSheet, Genorise Scientific, Inc) according to the manufacturer's instructions. Briefly, 96-well microplates were sensitized with primary antibody at room temperature for 30 minutes; the sample was added and incubated for 30 minutes at 37°C . After washing, the secondary antibody conjugated with peroxidase was added to each well and incubated. The concentration of the cytokines was

determined by the intensity of the color measured spectrophotometrically using a microplate reader.

The quantification of serum CRP was performed using commercial kits of ultrasensitive CRP (BioTécnica, Minas Gerais, Brazil) following the manufacturer's protocol at semiautomatic analyzer Bio-2000. Samples were treated with a specific antibody to horse CRP in a suitable buffer. The turbidity resulted by the formation of immune complexes was measured at 540 nm, and the values were then calculated automatically from a known standard. All the assay steps were performed automatically by spectrometry (BioTécnica).

Data were presented as mean values \pm standard deviation. Immunoglobulins, cytokines, and CRP levels results were subjected to Student test (t-test). Values with probability (P) less than 1% were considered statistically different.

3. Results

The results of the Igs, cytokines, and CRP levels are shown in Table 1. The IgG levels did not differ significantly between the groups, but IgM levels were significantly higher in seropositive horses for *Neospora* spp. compared with seronegative horses (Table 1). Similarly, levels of proinflammatory cytokines (TNF- α , IFN- γ , IL-1, IL-4, and IL-6) were higher in horses seropositive for *Neospora* spp., unlike IL-10 (anti-inflammatory cytokine), which showed lower levels compared with seronegative animals. Elevated levels of CRP were also observed in seropositive animals (Table 1).

4. Discussion

Several studies have reported the presence of antibodies to *Neospora* spp. in horses [14,16–19], but few studies have reported the protozoan infection with pathologic changes. As mentioned in Section 1, *Neospora* spp. infection was associated with equine abortion and/or stillbirths [11]. Inflammatory infiltrates have been described in placentas of cattle infected by *N. caninum*, which may be responsible for placental damage leading to abortion [20]. In buffalo, placental inflammation was characterized by the infiltration of CD3⁺ and CD4⁺ T cells and T cells expressing the $\gamma\delta$

Table 1

Immunoglobulins (IgG and IgM), cytokines (tumor necrosis factor [TNF- α], interferon gamma [IFN- γ], and interleukins [IL]: IL-1, IL-4, IL-6, and IL-10), and C-reactive protein (CRP) concentrations in horses seropositive to *Neospora* spp. compared with noninfected horses.

Parameters	Seronegative Horses ($n = 20$)	Seropositive Horses ($n = 23$)
IgG (mg/dL)	153.0 \pm 11.0 ^a	153.7 \pm 6.8 ^a
IgM (mg/dL)	55.0 \pm 6.0 ^b	121.6 \pm 5.5 ^a
TNF- α (pg/mL)	124.0 \pm 7.0 ^b	273.6 \pm 12.9 ^a
IFN- γ (μ g/mL)	152.4 \pm 4.0 ^b	312.6 \pm 20.9 ^a
IL-1 (pg/mL)	62.0 \pm 5.0 ^b	174.3 \pm 6.8 ^a
IL-4 (pg/mL)	39.2 \pm 5.2 ^b	147.6 \pm 8.8 ^a
IL-6 (pg/mL)	77.0 \pm 10.0 ^b	226.9 \pm 19.2 ^a
IL-10 (pg/mL)	72.0 \pm 7.0 ^a	46.0 \pm 6.8 ^b
CRP (mg/dL)	0.45 \pm 0.2 ^b	6.2 \pm 2.4 ^a

^{a,b} Means followed by the same superscript letters in the same line do not differ statistically ($P < .01$).

T-cell receptor, similar to cattle [21]. Probably the same inflammatory process occurs in pregnant mares infected with *Neospora* spp. leading to abortion [11]. In the present study, positive animals had no history of reproductive problems, but what keeps the immune response activated.

Seropositive horses to *Neospora* spp. showed high levels of all the cytokines evaluated in the present study, probably with the purpose of keeping the infection under control. In horses, in this study, probably the parasite is in the form of bradyzoites, in which the immune system requires constant surveillance of the host, as seen in this study. In horses naturally infected with *Trypanosoma vivax*, similar results were observed regarding the levels of inflammatory mediators studied here [22]. In a study of cell culture for *N. caninum*, researchers found that the IFN- γ , TNF- α , IL-10, and transforming growth factor- β cytokines participate in parasite proliferation and control mechanisms [23]. However, the vast majority of studies indicate that the IFN- γ is the main cytokine involved in the control of neosporosis and vertical transmission of this protozoan, as well as IL-4 [24–26]. Similarly, increased production of TNF- α , IL-1, and IL-6 contributes significantly to the course of infection and regulates specific immune responses against protozoan parasites [27], often causing tissue damage [28]. The low levels of IL-10 in seropositive animals were expected because it is an anti-inflammatory cytokine, that is, acts by inhibiting activated macrophages to produce proinflammatory cytokines. Therefore, if there were such negative feedback for IL-10, the levels of other mediators could be even greater in the present study.

In this study, only high levels of IgM in the serum of horses seropositive for *Neospora* spp. were found and IgG levels did not differ between groups. De Marez et al [29] compared the serum levels of IgG and IgM in calves experimentally infected by *N. caninum* oocysts, and levels of both Igs were detected 2 weeks after infection. Researchers found that bulls infected with *N. caninum* showed increased levels of IgG and IgM compared with healthy animals [30], but according to these researchers, IgG levels decreased with the chronicity of the infection. In our study, it is not possible to know the time of infection, but considering that IgG levels were not high, it might be a chronic infection. In infections by trypanosomes, IgM is responsible for controlling infection, that is, killing the parasite [31], and the same may occur in neosporosis, which would explain the high levels of IgM in seropositive horses.

Seropositive horses had elevated levels of CRP, an important acute phase protein and component of the immune system [32]. An increase of CRP levels has previously been observed during the course of diseases caused by other protozoa, such as infections by *Babesia canis* [33] and *Leishmania infantum* in dogs [34]. The main function of this protein is opsonization of the pathogen by activating the classical complement pathway and modulation of the action of monocytes and macrophages [32,35]. Therefore, this protein probably plays an important role in controlling infection by *Neospora* in horses and inhibits invasion of cardiac cells by *Trypanosoma cruzi* [36].

Based on the results, we can conclude that horses seropositive for *Neospora* spp. and visually asymptomatic have enabled humoral and cellular immunity. Increased

serum inflammatory mediators involved in the pathogenesis of this disease may be related to infection control, as well as that the inflammation can cause tissue lesions and thus be related to reproductive problems.

5. Commission of Ethics and Animal Welfare

This research project was approved by the Ethics Committee on Animal Experiments of Universidade do Estado de Santa Catarina under protocol number 1.05.09.

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