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Selected blood immunological and biochemical parameters in horses infected with *Cyathostominae* before and after ivermectin treatment

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Abstract: The objective of this study was to identify selected blood immunological and biochemical parameters in horses infected with *Cyathostominae* before and after treatment with ivermectin. Fecal samples were collected before ivermectin treatment and on days 1, 2, 3, 4, 10, 20, 30, 40, 50, 60, and 75 after drug administration. Blood was sampled on the same days to determine levels of total protein, γ-globulin, ceruloplasmin, and lysozyme activity. Eggs and dead parasites were excreted until day 3 postivermectin treatment. The reappearance of eggs was observed in 1 horse on day 50, and on day 75 in the remaining animals. Before treatment, total protein levels were measured at 53.2 g/L on average, and a decrease was noted until day 3 after treatment, with minor fluctuations on the following days. The highest γ-globulin concentrations of 14.1 g/L were reported on day 10 after treatment. Ceruloplasmin levels were determined at 74 IU before treatment, and a significant increase to 106 IU was observed on day 40. Lysozyme activity before ivermectin treatment reached 2.77 mL/L on average, and a significant 2-fold increase to 6.85 mg/L was reported on day 75.

Key words: Horses, *Cyathostominae*, ivermectin, immunology

1. Introduction

Infections with parasites of the subfamily *Cyathostominae* are widespread in horses, in particular in grazing animals where the disease is often symptom-free. The subfamily *Cyathostominae* includes 51 species of strongylid nematodes. These parasites have a simple life cycle. The eggs excreted by infected horses hatch into larvae, which become invasive after several days. Horses are infected per os by consuming larvae that colonize colonic and cecal mucosa. The parasites mature into adult forms or migrate to deeper layers of the intestinal wall, where they undergo hypobiosis. During primary infections, the prepatent period lasts from 8 to 20 weeks. In veterinary practice, such infections are difficult to diagnose and treat because the majority of drugs targeting internal parasites in horses are effective in eliminating adult nematodes only, and their efficacy is much lower against dormant larvae (1–4). Parasitological tests of horse feces only support determinations of the number of excreted eggs and evaluations of treatment efficacy based on the percentage reduction of excreted eggs. There are no effective methods to define the number of dormant larvae. Several attempts have been made to evaluate latent strongylid infections based on analyses of serum protein electrophoretic patterns (5,6), but the proposed method was regarded as insufficiently sensitive and specific (7,8). Lysozyme is an enzyme that plays an important role in the initial destruction of invasive agents and can serve as an early biomarker of inflammation (9). Ceruloplasmin is an acute phase protein whose concentration changes in response to different kinds of tissue damage and which therefore has been identified as the biomarker of choice for diagnostic and prognostic purposes in veterinary medicine. Evaluation of both parameters is considered to be indicator of the severity of the inflammatory process (10,11).

The objective of this study was to identify selected blood immunological and biochemical parameters in horses infected with *Cyathostominae* that change after treatment with ivermectin and during the reinfection period until the reappearance of eggs in the feces.

2. Materials and methods

The experiment was carried out from April to June 2010 on 24 horses. Feces samples were collected from the animals in the morning, directly from the rectum, and blood samples for biochemical analyses were obtained directly from the jugular vein.

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Feces samples were analyzed by the flotation method with the use of Darling's solution, as modified by McMaster (12). The number of parasite eggs per gram of feces (EPG) was counted for each horse. Eggs were identified according to the description given by Ziomko and Cencek (13) based on morphological analyses (shape, thickness and structure of the eggshell, number of blastomeres) performed under a light microscope at 400× magnification. Egg size was measured under the Olympus CX31 microscope with a digital camera, using the Quick PHOTO MICRO 2.3 application for image acquisition and visualization. To confirm the diagnosis of *Cyathostominae* infection, fecal samples were cultured and then the intestinal cells of the larvae were counted. All recovered larvae possessed 8 intestinal cells.

Upon the determination of *Cyathostominae* eggs in feces samples, the 9 horses excreting the highest number of eggs (250–700 eggs/g feces), aged 3 to 12 years, with body weight of 480 to 550 kg, were selected for successive parts of the study. Strongylid infection was treated with commercially available paste for oral application containing 1% ivermectin, in doses recommended by the manufacturer (0.2 mg/kg BW) and measured individually for each animal. The horses had ad libitum access to water and pasture throughout the experiment.

Feces and blood samples were collected 24 h after the administration of ivermectin and on treatment days 2, 3, 4, 10, 20, 30, 40, 50, 60, and 75. Around 200 g of feces was taken from each animal and placed into plastic containers. After mixing, 1 g of feces was collected from each sample on 3 occasions to determine parasite prevalence. The samples were analyzed, and the results were presented as the mean of 3 measurements.

Blood was sampled from the jugular vein into heparinized test tubes using a closed loop sampling system. The samples were centrifuged at 2500 rpm for 10 min at 4 °C. Plasma was poured into test tubes and stored at –80 °C until biochemical analysis.

Lysozyme activity in the plasma was measured by a turbidimetric assay (9). The assay is based on lysis of the lysozyme-sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Sigma), which is obtained freeze-dried from major chemical suppliers. A solution of *Micrococcus lysodeikticus* in a sodium phosphate buffer was mixed with plasma and incubated at 25 °C. Absorbance (450 nm) was measured before and 15 min after incubation in sterile plastic tubes. The standard was hen egg white lysozyme (Sigma).

Ceruloplasmin activity in the plasma was determined spectrophotometrically with modified fast micro-methods (14). Plasma was incubated in microplates for 15 min in an acetate buffer containing 0.2% p-phenylenediamine (PPD, Sigma). Sodium azide (0.02%) was used to stop the reaction. Ceruloplasmin activity was measured at 540 nm in a microreader (MRX 3 Dynatech).

Total protein and immunoglobulin (Ig) levels in the serum were determined using the Lowry micro method (Sigma, Diagnostic Kits). Total Ig concentrations were measured using the Lowry micro method as modified by Siwicki and Anderson (9). In the applied method, Ig is first precipitated from the serum with polyethylene glycol (10,000 kDa).

The results were processed by one-way ANOVA and Duncan's test at a significance level of $P \leq 0.01$ using the STATISTICA 9 application.

3. Results

Prior to treatment, an average of 450 strongylid eggs (250–700) were observed in 1 g of feces sampled from 9 selected horses. The oval eggs, measuring 90–122 × 45–70 µm, had thin walls and contained 8 to 16 dark, granular blastomeres, which are indicative of nematodes of the subfamily *Cyathostominae*.

Twenty-four hours after ivermectin administration, the EPG decreased significantly to reach 150 eggs (50–250) in 1 g of feces on average. On day 4 posttreatment, all of the analyzed feces samples were free of parasite eggs. The presence of strongylid eggs was monitored on successive days of the experiment. *Cyathostominae* eggs reappeared in 1 horse on day 50 and in 5 horses on day 60, and the presence of individual eggs was determined in the feces of all studied animals on day 75, as shown in Figure 1.

In biochemical analyses of blood samples collected before treatment, total protein (TP) level reached 53.2 g/L on average (49.7–58.5). TP concentration continued to decrease until the third day after ivermectin treatment, when it was 48.8 g/L (46.7–50.9). TP level increased on the fourth day after treatment, and it was marked by minor fluctuations on successive days of the experiment, reaching 52.8 g/L on average (50.2–57.7) on day 75, as shown in Figure 2.

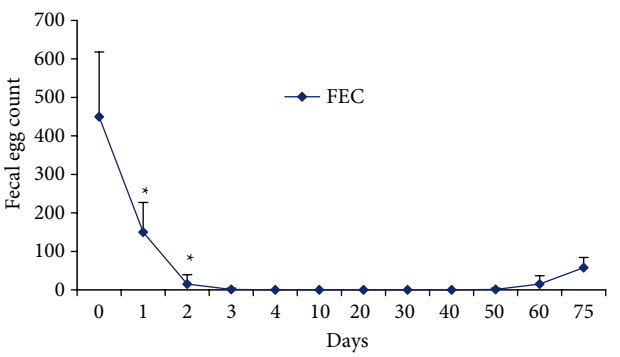

Figure 1. Average number of parasite eggs in 1 g of horse feces before and after ivermectin treatment.
(⊤ = standard deviation, * significantly different vs. day 0, $P \leq 0.01$)

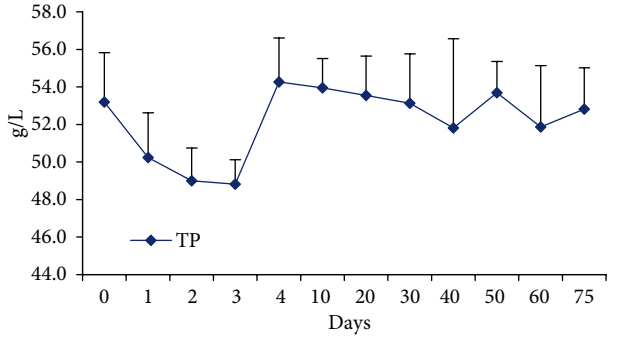

Figure 2. Average total protein levels in the blood plasma of horses before and after ivermectin treatment.
(┬ = standard deviation)

Before ivermectin treatment, average γ-globulin level was 11.6 g/L (8.4–17.1). On the third day after treatment, it decreased to 8.8 g/L (6.7–10.3), and an increase to 14.1 g/L (12.3–15.1) was reported on day 10. γ-Globulin concentration decreased steadily on successive days of the experiment to reach 10.7 g/L (7.9–13.3) on day 75, as shown in Figure 3.

Lysozyme activity before treatment was, on average, 2.77 mg/L (1.9–5.0). On the third day after treatment, it increased significantly to 5.38 mg/L (4.2–6.9). A significant increase in lysozyme activity was also reported on day 30 at 5.48 mg/L (4.2–7.2), and between days 50 and 75. On the last day of the experiment, lysozyme activity reached 6.85 mg/L on average (4.7–9.1), and it was 2-fold higher than on day 0, as shown in Figure 4.

Ceruloplasmin concentration reached, on average, 74 IU (66.8–82.2) before ivermectin administration. A significant increase was observed on the first day after treatment (90.2 IU). A successive increase in ceruloplasmin level was reported on day 4 at 88.2 IU (76.3–105.7). Ceruloplasmin level reached its highest value on day 40 at 106 IU (90–119.3), and remained significantly elevated until the end of the experiment, as shown in Figure 5.

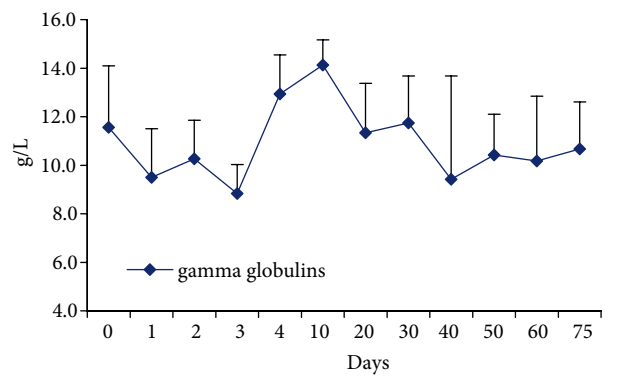

Figure 3. Average gamma globulin concentrations in the blood plasma of horses before and after ivermectin treatment.
(┬ = standard deviation)

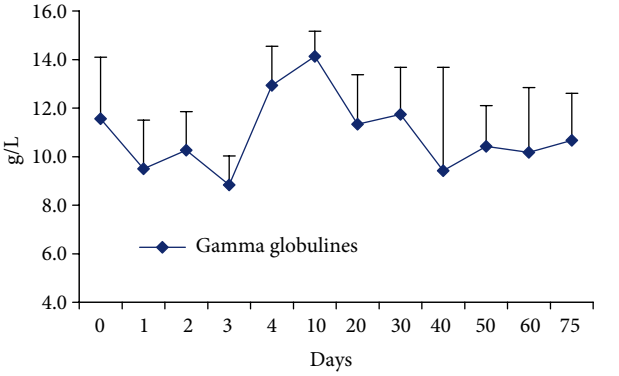

Figure 4. Average lysozyme activity in the blood plasma of horses before and after ivermectin treatment.
(┬ = standard deviation * significantly different vs. day 0, P ≤ 0.01)

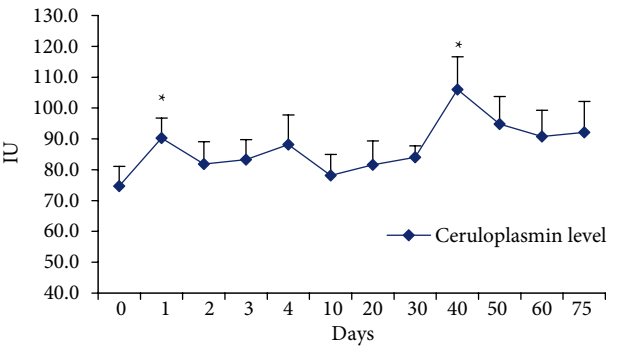

Figure 5. Average ceruloplasmin levels in the blood plasma of horses before and after ivermectin treatment.
(┬ = standard deviation, * significantly different vs. day 0, P ≤ 0.01)

4. Discussion

In horses, infections with parasites of the subfamily *Cyathostominae* are generally symptom-free, and the only observable disease signs include lower feed conversion and deteriorating health. There are no clear changes in blood biochemical or morphological profiles that could be regarded as pathognomonic signs of cyathostominosis, but neutrophilia and hypoproteinemia are observed in most infected horses (5,7,8,15). The above symptoms can be attributed to inflammation of intestinal mucosa. The most pronounced clinical symptoms are produced by fourth and fifth stage larvae returning to the intestinal lumen (1,7,16). The number of dormant larvae is difficult to estimate because they may survive in hypobiosis for up to 2 years (7,8) and because of the risk of constant reinfection while at pasture. Acute symptoms of larval cyathostominosis that resemble type II ostertagiosis in cattle are observed in early spring (7,17).

In the examined horses, average total protein levels did not reach the physiological norm (65–70 g/L) despite the

applied treatment and ad libitum pasture access. Based on the results of visual and clinical examinations, the animals appeared to be healthy and in good condition.

Low lysosyme activity and ceruloplasmin levels before treatment indicate the dynamic balance between the host and the parasite and show that horses in generally good health are able to compensate for the detrimental influence of small strongyles in the intestine. Increases in the ceruloplasmin concentration and levels of lysozyme activity on days 1 and 3 after treatment seem to be a result of lesions in intestinal mucosa associated with detachment of adult strongyles. In subsequent days the values decrease with mucosal healing. The observed increase in lysozyme activity and ceruloplasmin concentrations on days 30–40 after treatment points to the activation of the immune system in response to reinvasion and the activation of dormant larvae (4). Parasitic activity irritates and damages the walls of the large intestine, producing an inflammatory response, as shown by the results of blood biochemical tests. The above symptoms are observed in the prepatent period when parasitological examinations of feces produce negative results. Significantly lower ceruloplasmin concentration and lower level of lysozyme activity observed during the patent infection (before treatment) could suggest that the host's immune system has adapted to the presence of adult parasites in the intestine.

According to published data (2,4,18), the egg reappearance period (ERP) after ivermectin treatment lasts from 8 to 12 weeks. The results of our study corroborate the above findings. In horses infected with nematodes of the subfamily *Cyathostominae*, ivermectin treatment significantly reduces the number of eggs excreted with feces, but it does not completely eliminate dormant larvae, as demonstrated by the length of ERP, which is shorter than the prepatent period characteristic of primary *Cyathostominae* infection. The values of selected blood biochemical and immunological parameters fluctuate during infection caused by *Cyathostominae* nematodes, and they may offer an indirect estimation of mucosal damage caused by the parasites.

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