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Local and systemic inflammatory and immunologic reactions to cyathostomin larvicidal therapy in horses

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ABSTRACT

Encysted cyathostomin larvae are ubiquitous in grazing horses. Arrested development occurs in this population and can lead to an accumulation of encysted larvae. Large numbers of tissue larvae place the horse at risk for developing larval cyathostominosis. This disease complex is caused by mass emergence of these larvae and is characterized by a generalized acute typhlocolitis and manifests itself as a profuse protein-losing watery diarrhea with a reported case-fatality rate of about 50%. Two anthelmintic formulations have a label claim for larvicidal therapy of these encysted stages; moxidectin and a five-day regimen of fenbendazole. There is limited knowledge about inflammatory and immunologic reactions to larvicidal therapy. This study was designed to evaluate blood acute phase reactants as well as gene expression of pro-inflammatory cytokines, both locally in the large intestinal walls and systemically. Further, mucosal tissue samples were evaluated histopathologically as well as analyzed for gene expression of pro- and anti-inflammatory cytokines, cluster of differentiation (CD) cell surface proteins, and select transcription factors. Eighteen juvenile horses with naturally acquired cyathostomin infections were randomly assigned to three treatment groups; one group served as untreated controls (Group 1), one received a five-day regimen of fenbendazole (10 mg/kg) (Group 2), and one group received moxidectin (0.4 mg/kg) (Group 3). Horses were treated on day 0 and euthanatized on days 18–20. Serum and whole blood samples were collected on days 0, 5, and 18. All horses underwent necropsy with collection of tissue samples from the ventral colon and cecum. Acute phase reactants measured included serum amyloid A, iron and fibrinogen, and the cytokines evaluated included interferon γ, tumor necrosis factor α, transforming growth factor (TGF)-β, and interleukins 1β, 4, 5, 6, and 10. Transcription factors evaluated were FoxP3, GATA3 and tBet, and CD markers included CD163, CD3z, CD4, CD40, and CD8b. Histopathology revealed an inflammatory reaction with higher levels of lymphocytes, T cells, B cells, eosinophils and fibrous tissue in the moxidectin-treated group compared to controls or horses treated with fenbendazole. No apparent systemic reactions were observed. Expression of IL-5 and TGF-β in intestinal tissues was significantly lower in Group 3 compared to Group 1. This study revealed a subtle inflammatory reaction to moxidectin, which is unlikely to cause clinical issues.

1. Introduction

Cyathostomin parasites are ubiquitous in horses, and although most infected horses remain clinically normal, a severe disease syndrome, larval cyathostominosis, has been described. This is characterized by diarrhea caused by a generalized typhlocolitis resulting from the synchronous emergence of cyathostomin larvae from the large intestinal mucosa (Love et al., 1999). Cyathostomin larvae are known to undergo arrested development at the early third larval stage (EL3) and can accumulate in populations that reach hundreds of thousands. More mature larval stages include late third (LL3) and fourth (L4) stages, which remain encysted until seasonal factors or changes in population structure stimulate them to excyst and make their way to the intestinal lumen. Larval excystment has been associated with a pronounced inflammatory reaction when large numbers of larvae emerge synchronously. Removal of the luminal cyathostomin stages by adulticidal anthelmintic treatment is believed

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to trigger mass emergence of encysted cyathostomin larvae and cause acute inflammation of the intestinal walls (Love et al., 1999). Although uncommon, larval cyathostominosis has a 50% case-fatality rate and, paradoxically, anthelmintic treatment has been identified as a significant risk factor (Reid et al., 1995).

Only two currently marketed equine anthelmintics have label claims for efficacy against equine encysted cyathostomins: moxidectin gel (0.4 mg/kg) administered once orally, and fenbendazole (10 mg/kg) administered orally once daily for five consecutive days. However, it is well documented that anthelmintic resistance to adulticidal doses of benzimidazoles is widespread (Kaplan et al., 2004; Lester et al., 2013; Relf et al., 2014). Despite widespread acceptance that fenbendazole may no longer have satisfactory adulticidal efficacy, an annual five-day larvicidal fenbendazole treatment at 7.5 or 10 mg/kg/day is still widely used as a distinct element of a parasite management program on many farms. However, this approach has failed to achieve acceptable egg count reduction in several studies (Chandler et al., 2000; Rossano et al., 2010; Mason et al., 2014). In addition, recent studies have reported a marked decrease of the egg reappearance period following moxidectin treatment (Rossano et al., 2010; Lyons et al., 2011; Relf et al., 2014), and this has been found to be associated with survival of luminal L4 cyathostomin larvae (Lyons et al., 2010). However, it remains unknown whether these developments may affect the larvicidal efficacy of moxidectin.

While larval cyathostominosis is well described, the local inflammatory and immunologic mechanisms associated with encysted and emerging larvae are poorly understood. One study illustrated that numbers of arrested EL3 as well as LL3s and L4s correlated significantly with local mucosal interleukin (IL)-4 and IL-10 production (Davidson et al., 2005). To date, only one study has evaluated local inflammatory responses to larvicidal therapy with fenbendazole or moxidectin (Steinbach et al., 2006). This study reported a significantly reduced local cellular inflammatory response in ponies treated with moxidectin compared to the five-day fenbendazole regimen or to untreated controls (Steinbach et al., 2006). These findings were based on histopathology whereas local cytokine production has not yet been evaluated in response to larvicidal anthelmintic treatment.

The systemic acute phase response to adulticidal anthelmintic therapy has recently been characterized in ponies and was found to be subtle and primarily associated with a fibrinogen response (Nielsen et al., 2013). However, similar studies have not been conducted with larvicidal anthelmintic treatment regimens. Another study evaluated blood leukocyte expression of pro-inflammatory cytokine genes in ponies undergoing anthelmintic treatment and found interleukin IL-1β to increase in response to both fenbendazole and pyrantel pamoate treatment, and IL-6 and tumor necrosis factor (TNF)-α to increase in response to a larvicidal regimen of fenbendazole (Betancourt et al., 2014). In contrast, no responses were observed to moxidectin treatment (Betancourt et al., 2014). Taken together, the current body of admittedly meager evidence suggests that the inflammatory reaction following moxidectin treatment is negligible.

The objective of this study was to characterize local and systemic immunological and inflammatory reactions to larvicidal therapy in a cohort of horses naturally infected with cyathostomins.

2. Materials and methods

2.1. Experimental design

This study was approved by East Tennessee Clinical Research's Institutional Animal Care and Use Committee, protocol number ETCR-14-0134. A masked, randomized, controlled clinical study was conducted in juvenile horses at a single site to evaluate the efficacies of a five-day regimen of fenbendazole oral paste (10 mg/kg) or a single, oral treatment with moxidectin gel (0.4 mg/kg) against luminal and mucosal stages of a known fenbendazole-resistant cyathostomin population. Anthelmintic efficacy results are reported in a separate publication (Reinemeyer et al., submitted).

Beginning on 13 February 2014, qualified candidates were housed in individual stalls and acclimated to study conditions for seven days. Horses were blocked by prior anthelmintic history, ranked within block by decreasing magnitude of strongylid egg count, and allocated randomly to one of three groups. After animals had been assigned to numbered groups, one of three treatments was allocated randomly to a group number. Group 1 horses ($n = 6$) served as untreated controls. Group 2 horses ($n = 6$) were treated once daily on days 0 through 4 with doses of Safeguard paste (Merck Animal Health, Summit, NJ, USA) at 10 mg fenbendazole/kg body weight. Group 3 horses ($n = 6$) were treated once on day 4 with a measured dose of Quest Gel (Zoetis Inc., Kalamazoo, MI, USA) at 0.4 mg moxidectin/kg body weight.

Blood and fecal samples were collected before administering the anthelmintic (day 0) and 7 and 14 days after the final dose of fenbendazole (i.e., on days 11 and 18). Blood samples were collected in serum-separator evacuated tubes, citrate-stabilized tubes, and Tempus blood RNA tubes (Life Technologies, Grand Island, NY, USA). Beginning on day 18, complete replicates of horses were euthanatized and necropsied for determination of total worm counts. Equal numbers of animals from each treatment group were processed on the same day. At necropsy, the cecum and colon were excised from each horse and weighed. Two replicates of full-thickness 10 mm × 10 mm samples were collected from each organ. One replicate was stored in 10% buffered neutral formalin, while the other was immediately transferred to RNA later (Life Technologies, Grand Island, NY, USA) and refrigerated.

2.2. Acute phase inflammatory markers

Serum samples were left to coagulate at room temperature for 24 h prior to centrifugation at 1000 × *g* for 10 min. Plasma samples were similarly centrifuged immediately upon collection. Plasma and serum were pipetted into cryotubes and stored at −80 °C for up to 8 weeks until shipped on dry-ice to the University of Copenhagen, where the following analyses were performed: serum iron concentrations by colorimetric spectrophotometry (ADVIA 1650, Bayer A/S), serum amyloid A (SAA) concentrations by immunoturbidometry (LZ test SAA; EIKEN Chemical Company) (Jacobsen et al., 2006), and fibrinogen concentration by the Clauss method in an automated coagulometric analyzer (ACL 9000, Instrumentation Laboratory Barcelona) (Pihl et al., 2013).

2.3. Cytokine gene expression

2.3.1. Blood samples

The filled Tempus tubes were immediately inverted 8–10 times, incubated at room temperature for 24 h, and then stored at −20 °C until further analysis. Gene expression was assayed using previously described techniques (Nielsen et al., 2013) for the following cytokines: interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukins IL-6, IL-10, and IL-1β. Intron-spanning TaqMan primers and probes for each target were obtained from Life Technologies and had been validated by the company. PCR amplification efficiencies were determined using Linreg (Ramakers et al., 2003) and only samples with amplification efficiencies above 99% were included for further analyses. Beta-glucuronidase (β-GUS) was used as the housekeeping gene. The pre-challenge sample for each horse used as the calibrator and the relative quantity (RQ) for