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Research paper

Evaluation of the mucosal inflammatory responses to equine cyathostomins in response to anthelmintic treatment

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ABSTRACT

Members of Cyathostominae are pervasive parasites of equids that can cause larval cyathostominosis, a potentially life-threatening disease that occurs when a multitude of encysted larvae synchronously excyst from the wall of the large intestine. Moxidectin and fenbendazole are the two current labeled drugs that target the encysted larval stages; however, there is limited knowledge of the local inflammatory response to the larvae and to the two treatments in clinically healthy horses. This study is the first to evaluate the local inflammatory response to cyathostomin larvae and to larvicidal treatment at 2 and 5 weeks post treatment. Thirty-six ponies with naturally acquired cyathostomin infections were randomly allocated into 3 groups: Group 1, fenbendazole at 10 mg/kg for 5 days, Group 2, a single dose of moxidectin at 0.4 mg/kg, and Group 3, untreated controls. Tissue samples from the cecum and dorsal and ventral colons were used for histopathological and immunohistochemical evaluation. Tissues were stained with routine hematoxylin and eosin (H&E) for light microscopy and immunohistochemically for MAC387, CD20, and CD3 for differentiation of activated macrophages, B cells, and T cells, respectively. Semiquantitative scores were assigned for all inflammatory cell types and fibrous connective tissue. Larvae observed by light microscopy were enumerated and classified by stage. Mucosal ulcerations and submucosal granulomas were also enumerated. Mean macrophage scores were higher in the moxidectin group than the fenbendazole group ($p = 0.0185$) and the control group had a higher activated macrophage score than both treatment groups ($p = 0.0104$, $p = 0.0004$). T lymphocyte scores were higher in the moxidectin group when compared to the control group ($p = 0.0069$). Goblet cell hyperplasia scores were elevated at 5 weeks post treatment compared to 2 weeks post treatment ($p = 0.0047$) and were elevated in the ventral colon compared to the dorsal colon ($p = 0.0301$). Eosinophil scores were elevated surrounding degenerative larvae when compared to intact larvae ($p = 0.0001$). Mucosal ulcerations were found only in the control group at 2 weeks post treatment. This study found subtle inflammatory differences between treatment groups but provided new information about goblet cells and eosinophils in relation to encysted cyathostomin larvae.

1. Introduction

Equine cyathostomin parasites are omnipresent in grazing horses across the world, and infection is virtually inevitable. This group of parasites consists of 14 genera and 50 species, of which 8 genera and 40 species are described infecting horses (Lichtenfels et al., 2008). The life cycle is unique among strongylids, as cyathostomin larvae are known to undergo arrested development at the early third larval stage (EL3) (Eysker et al., 1984), where hundreds of thousands of larvae can accumulate over time, presumably under the influence of a host response to the invading larvae (Chapman et al., 2003). Furthermore, EL3 counts have been reported to be significantly higher during seasons characterized by weather conditions that are unfavorable for parasite transmission on pasture (Ogbourne, 1975; Eysker et al., 1990; Chapman et al., 2003). Larvae eventually mature into late third (LL3) and fourth (L4) stages before they leave their cysts and make their way back to the intestinal lumen. This process has been associated with a pronounced inflammatory reaction, and when large numbers of larvae emerge synchronously, it can cause a severe typhlocolitis known as larval cyathostominosis (Love et al., 1999). While the disease complex is well-described, the local inflammatory and immunologic mechanisms are poorly understood.

Abbreviations: H&E, hematoxylin and eosin stain; EL3, early third larval stage; LL3, late third larval stage; L4, fourth larval stage; CD, cluster of differentiation; L3, third larval stage; MUC5AC, Mucin 5AC; RELM-β, resistin-like molecule beta; FCT, fibrous connective tissue; VC, ventral colon; DC, dorsal colon

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Of the current marketed equine anthelmintics, only fenbendazole (10 mg/kg or 7.5 mg/kg) administered orally once daily for five consecutive days and moxidectin gel (0.4 mg/kg) administered once orally are labeled for use against encysted cyathostomins. Of the two treatments, cyathostomins have documented resistance to the adulticidal dose of fenbendazole, as well as the other benzimidazoles (Kaplan et al., 2004; Lester et al., 2013) and the larvicidal dose regimen (Reinemeyer et al., 2015; Bellaw et al., 2018).

When considering the inflammatory response to treatment, one study suggested a significantly reduced local inflammatory response in ponies treated with moxidectin compared to the five-day fenbendazole regimen (Steinbach et al., 2006). We recently evaluated local and systemic inflammatory markers in response to moxidectin and the larvicidal regimen of fenbendazole and found no systemic responses to any of the two treatments (Nielsen et al., 2015). Locally, subtle inflammatory reactions were associated with moxidectin treatment but not fenbendazole treatment; however, this was largely contributed to the reduced larvicidal efficacy of fenbendazole (Nielsen et al., 2015).

Recently, we completed another treatment trial comparing the larvicidal effects of the five-day fenbendazole treatment regimen (n = 12) with moxidectin (n = 12) and an untreated control group (n = 12). In addition, to comparing the larvicidal efficacy of these two anthelmintic formulations, we also evaluated two different time intervals between treatment and necropsy; 2 weeks and 5 weeks. Anthelmintic efficacy results from this study are presented elsewhere (Bellaw et al., 2018). Overall, mucosal worm burdens were substantially higher than in the previous study (Reinemeyer et al., 2015), but the 2-week post treatment larvicidal efficacies were very similar with reduced larvicidal efficacy of the fenbendazole regimen (50.4%), whereas moxidectin efficacy (73.8%) was within historically reported ranges (Bellaw et al., 2018).

The aims with the present study were to collect further histopathological information on local inflammatory responses to anthelmintic therapy and perform the first comparison of histopathological reactions observed at the two different time intervals post anthelmintic treatment.

2. Materials and methods

2.1. Study design

The University of Kentucky's Institutional Animal Care and Use Committee approved this study, protocol number 2015–2092. The study was carried out between September and October of 2015. Thirty six ponies ranging from two to four years of age with naturally acquired cyathostomin infection were kept on pasture throughout the study. Ponies were blocked into groups of three based off age and decreasing magnitude fecal egg counts (performed with mini-FLOTAC, detection limit of 5 eggs per gram (Barda et al., 2014)). One pony from each block was randomly allocated into one of three treatment groups: fenbendazole treated, and moxidectin treated, and untreated control groups. Twelve ponies were allocated into each of the 3 treatment groups. All study personnel were blinded to group allocation and treatments throughout the study.

On Day-1, each pony was weighed on a certified livestock scale and individual doses of larvicidal anthelmintic were prepared based off weight and group assignment. Ponies assigned to the fenbendazole treatment group received 10 mg/kg fenbendazole (Panacur PowerPak, Merck Animal Health, Madison, NJ, USA) for five days, Days 0–4. Ponies assigned to the moxidectin treatment group received 400 μg/kg of moxidectin (Quest, Zoetis, Kalamazoo, MI) orally once on Day 4. On Days 18 and 19 (2 weeks post treatment), three ponies randomly selected from each group were necropsied and tissues samples taken. Three complete replicates were necropsied each day to ensure that equal numbers of ponies from each treatment group were necropsied simultaneously. The rest of the 18 ponies were kept on pasture until Days 35 and 36 (5 weeks post treatment), when they were euthanized and tissue samples were taken.

2.2. Histology

Tissue samples of approximately 2 × 5 cm were collected from grossly normal appearing middle portions of the cecum, ventral colon and dorsal colon. The samples were stored in 10% formalin for 24 h then transferred to 70% ethyl alcohol until use for histopathology purposes. These tissue samples were routinely processed and stained with Harris hematoxylin and eosin. All samples were analyzed by light microscopy and immunohistochemistry as previously described (Nielsen et al., 2015). In summary, larvae were evaluated based on size and morphological characteristics to determine larval stage (L3 or L4). Lesions associated with 3rd and 4th stage cyathostomin larvae were counted and histologically assessed for location (colonic glands, mucosal epithelium, lamina propria, or submucosa), size, inflammatory cell composition (neutrophils, eosinophils, macrophages, and lymphocytes), and severity of inflammation and fibrosis (0 = none; 1 = 1–5 cell layers, mild inflammation or fibrosis; 2 = 6–15 cell layers, moderate inflammation or fibrosis; or 3 = > 15 cell layers, severe inflammation or fibrosis). Additionally, tissue sections were analyzed for the presence or absence of goblet cell hyperplasia, mucosal ulceration, and submucosal granulomas.

2.3. Immunohistochemistry

Immunohistochemistry was utilized to characterize the mononuclear leukocyte population. Tissue sections were immunohistochemically stained (Dako, EnVision + Dual Link System-HRP) for Cluster of differentiation 3 (CD3) (Dako polyclonal rabbit anti-human CD3, Code A0452), CD20 (Thermo Scientific rabbit polyclonal antibody, Catalog #RB-9013), and MAC387 (Dako monoclonal mouse anti-human Myeloid/Histiocyte antigen, clone 387) to quantify T cells, B cells, and activated macrophages, respectively. The number of immunohistochemically stained cells were semiquantitatively graded (0 = none; 1 = 1–5 cell layers; 2 = 6–15 cell layers; or 3 = > 15 cell layers) for the radius of each parasite associated inflammatory focus.

2.4. Statistical analyses

All statistical analyses were carried out using SAS University Edition (SAS Institute, Cary, NC, USA). Generalized mixed linear models were developed to analyze the effects of treatment groups and weeks post treatment on the histopathological findings. Inflammatory nodule radius size and total mucosal length were the only continuous variables, while all others were labeled as categorical variables. Horse ID was kept as a random effect. Models were generated to analyze cell counts, larval counts, goblet cell hyperplasia, mucosal ulcerations, and inflammatory nodules with and without parasites. L3s, L4s, and degenerative larvae were evaluated in the cecum, ventral colon, and dorsal colon in respect to their locations in the submucosa, lamina propria, and mucosa. For all, 'organ' and 'location' were covariates. Total length of the organ mucosa of all samples of all groups was analyzed for possible bias as well. Influence of all measured parameters for each analysis was evaluated using traditional backward and forward elimination of variables. All variables with p-values < 0.25 were kept in the model. The interaction between 'groups' and 'weeks post treatment' was also evaluated in each analysis. When variables were significant, a 'least square means' for a Tukey's pairwise comparison, odds ratio, and estimate were all performed and interpretation of results occurred at the significance level $\alpha = 0.05$.