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Research paper

Encysted cyathostomin larval counts: Mucosal digestion revisited

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ABSTRACT

Cyathostomins are pervasive equine parasites in horses across the world, and larval stages are known to cause the deadly disease larval cyathostominosis. The mucosal digestion technique is widely used for enumeration of encysted larval stages. Previous studies have investigated the spatial variation of encysted larvae, however current protocols lack a description of a standardized area from which to take the tissue sample. This study sought to evaluate spatial variation in encysted cyathostomin larval counts among the large intestinal organs and their subsections. Following humane euthanasia, ceca, ventral, and dorsal colons were harvested from 8 foals (aged 4–8 months) raised in an anthelmintic naïve parasitology research herd. Each organ was weighed and separated into 3 equal sections by length: the orad, intermediate, and aborad portions. From each of those sections, two 5% weight tissue samples were collected and digested to quantify the early third stage larvae (EL3) and late third stage larvae/fourth stage larvae (LL3/L4). A mixed model statistical analysis was carried out to evaluate for differences of larval counts among the different organs, sections, and the interaction term between the organs and sections. There were significant differences among organs ($P < 0.0001$), with the ceca having higher counts than the ventral and dorsal colons. However, there were no significant differences among the three defined organ sections ($P = 0.1076$). Coefficients of variation (CV) were all calculated to be greater than 1, suggesting a high level of variability among the samples; the least amount of variation can be found in the cecal data with a CV of 1.4024 compared with the ventral colon's 1.529845 and dorsal colon's 3.339135 within the respective organ. The following sections had the highest mean counts of encysted larvae: intermediate cecum, orad ventral colon, and aborad dorsal colon. Though only a portion of the results were significant, trends were observed and these should be investigated further in future studies and potentially employed in larvicidal efficacy evaluations.

1. Introduction

Equine cyathostomin parasites are ubiquitous in grazing horses around the world. Their life-cycle includes tissue-dwelling stages in which early third stage (EL3) develop sequentially into late third stage (LL3) and mucosal fourth stage (L4) larvae which encyst in the mucosa of the cecum, ventral colon, and dorsal colon. The EL3s can undergo arrested development for variable durations of up to two years (Gibson, 1953; Smith, 1976). Climatic differences also can lead to a discrepancy in EL3 arrestment. Climatic differences result in variations in seasonality and pasture infectivity, leading to an accumulation (or lack thereof) of encysted EL3s, which is believed to increase the risk of parasitic disease (Leathwick et al., 2019a, 2019b).

Cyathostomin-associated disease occurs when there is a mass emergence of encysted larvae, causing the condition known as larval cyathostominosis (Love et al., 1999), and the most common symptoms are diarrhea and sudden weight loss. There is a seasonal trend in temperate climates, with more cases occurring in the winter months (Reid et al., 1995). Horses less than five years of age (Giles et al., 1985) and dewormed within the past 2 weeks are more likely to experience larval cyathostominosis (Reid et al., 1995). In severe cases death may result with a reported case-fatality rate of about 50% (Reid et al., 1995).

No ante-mortem diagnostic tests are currently available for detecting and enumerating encysted cyathostomin burdens. Two post-mortem techniques have been described for enumerating encysted cyathostomin burdens; transmural illumination and artificial digestion (Chapman et al., 1999; Reinemeyer and Herd, 1986a). While both techniques have been found useful for estimating the abundance of the later developing stages (LL3 and L4), it has been demonstrated that the transmural illumination technique is insufficient for estimating EL3 burdens (Chapman et al., 1999). Thus, the mucosal digestion technique is widely used in research studies as it allows identification and enumeration of all larval stages (Eysker et al., 1984). Therefore, this technique remains the only method to evaluate larvicidal efficacy of

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anthelmintic products and studying cyathostomin parasite dynamics. However, despite its wide usage, current protocols lack standardization. Typically, a 5% weight subsample is collected from each of the large intestinal organs, digested, and diluted to a 20% mixture. From the mixture a 2% aliquot is examined and a multiplication factor is applied to achieve an estimate of total encysted burdens (Reinemeyer and Herd, 1986a). It should be mentioned that the percent aliquot examined can more greatly influence the multiplication factor than the percentage of tissue digested initially. But the location of the 5% subsample within the intestinal organ is usually not specified. One previous study provided some estimation of the variability within the same digest (Chapman et al., 1999) and another study showed variability within each organ using a transillumination technique for enumeration of larvae (Reinemeyer and Herd, 1986b). Given the continued need for using this technique for studying the pathogenic larval stages of equine cyathostomin parasites, it is important to evaluate these aspects.

The aims of this study were to use the mucosal digestion technique to 1) evaluate the variability of larval counts among the three large intestinal organs, and 2) to evaluate the variability of larval counts among various locations within the same organ.

2. Materials and methods

This study was performed from October 1, 2018 to December 12, 2018 at the Gluck Equine Research Center in Lexington, Kentucky, USA. Eight mixed-breed horse foals from an anthelmintic naïve research herd were used for sample collection (Lyons et al., 1990). Foals were humanely euthanized and necropsied between four and eight months of age. The study was conducted under approval from the University of Kentucky's Institutional Animal Care and Use Committee under protocol 2012-1046.

2.1. Sample collection

At necropsy, whole and emptied large intestine organ sets were collected and separated into cecum, ventral colon, and dorsal colon. The orad end of each organ was identified with a clamp to ensure correct orientation during measurement. The organs were weighed and measured longitudinally. Each organ was then subsectioned into three equal-sized portions: the orad, intermediate, and aborad sections. Although the cecum is a blind sac structure, for ease and clarity of the paper, the orad sections correspond to the base and the aborad sections correspond to the apex. From each of these defined sections, two full-length samples representing 5% of the total organ weight were excised longitudinally between the taenial bands and trimmed until the target weight was achieved. Two separate 5% weight strips were cut from each subsection and digested, allowing for replicates.

2.2. Mucosal digestion

The mucosal layer from the 5% strips were then scraped from underlying tissues with a glass microscope slide and placed in a correspondingly labelled screw cap flask. Each tissue sample varied in size due to age of foal and level of inflammation. It should be noted that no mucosal sample ever exceeded 50 mL. Each flask was then filled to 200 mL with a digestion solution comprised of 10 g pepsin, 1 L of warm water, and 15 m L of HCl. Each sample was digested for two hours at 37 °C with constant agitation, as previously described (Nielsen and Lyons, 2017). Although larger scrapings could have been digested longer and smaller samples for less time, it was more pragmatic to digest all of the samples at an equal duration. Digesting for longer durations could affect the quality of the larvae for identification as eventually the specimens would be susceptible to digestion themselves. The second set of tissues were scraped and placed into specimen jars with respective labelling and refrigerated at 4 °C for no more than 36 h. After the first set was digested, the stored set was scraped and digested using the same protocol.

2.3. Mucosal larval enumeration

A 2% aliquot of the digested mucosa was examined at 40x for identification of larval cyathostomins. Larvae were counted and simultaneously identified as EL3 or LL3/L4 (collectively designated as developing larvae) using morphological criteria (Nielsen and Reinemeyer, 2018).

2.4. Data analyses

All statistical analyses were performed in SAS version 9.4 (Cary, NC, USA). A mixed model was constructed to identify differences in mucosal larval counts using sex (male or female), age (4–5 months, or 6–8 months), organ (cecum, ventral colon, or dorsal colon), section of organ (orad, intermediate, and aborad), and organ weight (< 1600 g, 1600–2399 g, > 2400 g) as covariates. An interaction term between organ and section was evaluated and tissue replicate was kept as random effect. Results were interpreted at the 0.05 significance level.

Coefficients of variation (CV) were calculated for each organ and section to evaluate the spatial variation of mucosal larval counts.

3. Results

Due to sparsity of of EL3 larvae in the data set, all larval counts were combined. The mean mucosal larval counts are represented in Fig. 1 Fig. 1. Significantly higher counts were obtained in the cecum compared with the other two organs ($P < 0.0001$), whereas no significant differences were observed among organ subsections ($P = 0.1076$), and no interaction between the organ and subsections was observed ($P = 0.1281$).

The coefficients of variation throughout the data set were greater than 1 with the exception of the cecum apex section, as seen in Tables 1 and 2. There was no effect of refrigeration on the tissue samples ($P = 0.7873$). There was no effect of foal age, with 'young' foals (4–5 months-of-age) similar to 'old foals' (6–8 months-of-age) ($P = 0.6759$), nor was there an effect of sex ($P = 0.2515$).

4. Discussion

This is the first study to examine the mucosal digest procedure with a purpose of evaluating spatial influence within intestinal organs on estimates of encysted cyathostomin larval burdens. Similar findings were made in a previous study conducted with the transillumination technique (Reinemeyer and Herd, 1986b), although in the current study there was no significant differences between organ sections. Perhaps more importantly, substantial variation in the mucosal larval counts was observed in the current study which needs to be taken into account when interpreting worm burden data.

The large variability between subsamples is likely the explanation why no statistically significant differences were observed between organ sections. However, with p-values around 0.10 it cannot be ruled out that statistical significance could have been achieved with a larger data set. It should be acknowledged that foals generally have relatively low numbers of encysted cyathostomin larvae (Nielsen and Lyons, 2017), as well as a deficient level of EL3 encystment, therefore all larval counts have been combined into a total count. This likely contributed to the high CV values reported herein. This is supported by the standard deviations reported in a previous study of naturally infected ponies (Chapman et al., 1999), where encysted burdens were substantially greater than reported here, and the standard deviations relatively smaller. Ranging from 1.9 to 69.3, the standard deviations reported by Chapman et al. were dwarfed by this study's calculations in the hundreds. Thus, it would be of value to study spatial variation in more mature horses with larger encysted burdens to evaluate whether the

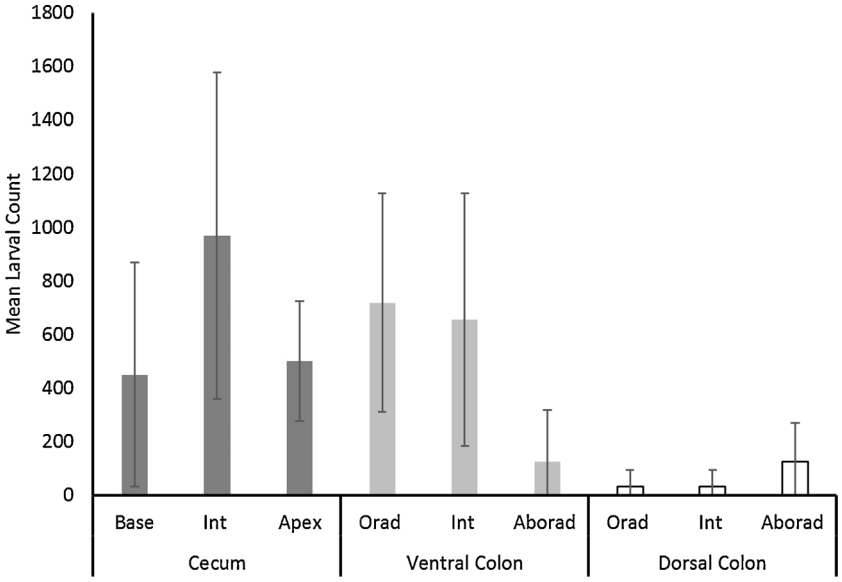

Fig. 1. Mean mucosal cyathostomin larval counts by intestinal compartment. "Base" and "orad" distinguish the sections of the organ closest to the mouth, "Int" distinguishes the middle length of the organ, and "Ab" or "Ap" distinguish the length of the organ nearest the rectum. The error bars are set at a 95% confidence interval.

Table 1
Coefficients of variation (CV) for mucosal larval counts determined for each large intestinal organ. Variation coefficients greater than 1 indicate standard deviations greater than the mean for the organ.

Organ	CV
Cecum	1.40
Ventral Colon	1.53
Dorsal Colon	3.34

Table 2
Coefficients of variation (CV) for mucosal larval counts determined for each organ section. Variation coefficients greater than 1 indicate standard deviations much greater than the mean for a section.

Organ Section	CV
Cecum Base	1.85
Cecum Intermediate	1.26
Cecum Apex	0.89
Ventral Colon Orad	1.14
Ventral Colon Intermediate	1.44
Ventral Colon Aborad	3.10
Dorsal Colon Orad	4.00
Dorsal Colon Intermdiate	4.00
Dorsal Colon Aborad	2.31

trends observed in the present study could represent true differences. More mature horse populations will have stronger immune systems as well as a larger proportion of encysted larvae due to the arrestment of EL3 larvae in the mucosae (Chapman et al., 2003; Nielsen and Lyons, 2017).

The cecum had the greatest mucosal larval population contrasted by the lower counts in the dorsal colon. Previous studies also found counts to be significantly lower in the dorsal colon, although counts from the cecum and ventral colon were not significantly different (Reinemeyer and Herd, 1986b; Nielsen and Lyons, 2017). Overall, this study confirmed that the dorsal colon only harbors a small proportion of the overall population of encysted larvae. Nonetheless, it appears advisable to still include the dorsal colon in studies aiming at estimating the encysted cyathostomin burden.

We used a standard 5% subsample in this study, and larger subsamples could very likely have reduced data variability. However, the mucosa to digestion fluid ratio is critical and it is commonly practiced to not exceed 25 g of tissue in 200 ml of digestion solution (Chapman et al., 1999), as there is an upper limit to how much tissue can be harvested for a given digest, especially with larger horses. In foals, however, intestinal organs are relatively small in size, so digesting a larger subsample could still be feasible and would be worth a consideration. If using larger tissues samples was not feasible, using a larger aliquot from the digestion solution would also lead to reduced variability. Performing the digestions in duplicate, as done in the present study, will also reduce variability, and, hence, increase precision of the estimate, but it will be more time-consuming and require a higher shaker-incubator capacity for studies where samples from several horses are to be processed within the same day. Storing samples for digestion in subsequent days is one way to address such logistical challenges. The refrigeration of mucosal subsamples for up to 36 h in this study is unlikely to have affected the data as a previous study found that formalin or ethanol-glycerin fixation and storage at -20 °C did not affect encysted counts (Chapman et al., 1999). There was no significant difference between the fresh and refrigerated samples.

In this study, a whole-animal experiment was the only possible approach for this study as no *in vitro* alternatives exist for evaluating spatial variation and general variability in cyathostomin larval counts. Terminal controlled efficacy studies remain the gold standard for anthelmintic efficacy determination in equine parasitology, so it remains important to study standard techniques employed and seek ways to improve the quality of the data obtained.

In conclusion, this study demonstrated that cyathostomin larvae have organ preferences in naturally infected foals with higher numbers recovered from the cecum. Furthermore, we demonstrated high levels of variability between repeated counts from the same organs. Trends of spatial variation were observed, but they were not statistically significant in the data material at hand. Further studies in older age groups are required to fully explore the spatial variation of mucosal cyathostomin larval counts.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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