OPTIMIZATION OF QUANTITATIVE TECHNIQUES FOR ENUMERATING MUCOSAL STAGES OF CYATHOSTOMIN NEMATODES IN THE HORSE

by

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ABSTRACT

In researching equine parasites, larval cyathostomin populations must be quantified. Mechanical digestion was developed over 30 years ago to enumerate encysted stages of cyathostomes. This study examined the optimal digestion times for liberating early stage L3 larvae (EL3) and late stage L3 and L4 larvae (collectively known as DL). Mucosal samples from both the cecum and large intestine were digested for 60, 90, 120 or 150 min. A time effect ($p = 0.032$) was observed for EL3 in the cecum, with the greatest number at 150 min. The ventral colon showed a similar time effect ($p = 0.035$) being greatest at 150 min. There was no effect on DL from 60-150 min in either the cecum ($p = 0.71$) or ventral colon ($p = 0.67$). Longer time intervals should be examined to determine the optimum time for liberating EL3, while a different test for DL is recommended.

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INTRODUCTION

Managing parasites is an important part of animal husbandry for any livestock species. In the horse, internal parasites may be detrimental to both health and animal performance. However, many tactics utilized for treating as well as studying equine parasites are quite antiquated. The development of new techniques and treatments can benefit the health and well-being of horses worldwide. However, before developing such tactics, it is important to understand internal parasites in the horse, with a particular focus on cyathostomes.

Treatments of internal parasites have varied over the years from blood-letting to current anthelmintics. In the 1600s a veterinarian named Markham mentioned in his writings that he would "let the blood" and then have the horse drink it to purge the parasites. He also mentioned that he would not treat pregnant mares because treatments could harm the baby. They diagnosed parasite burdens by bad breath, a slimy mouth, and "worm-veins" under the lips. Other methods of treatment through the centuries include: hen and pigeon guts, hen eggs, hen and human feces, licorice, linseed, black soap, and quicksilver. Products such as these remained until the beginning of scientific testing in the 1900s. The critical test where the horse was used as its own control began in 1917. They treated the horse and then put it down and counted the parasites to determine treatment efficacy. These tests continued, as well as some field tests, to develop antiparasitic compounds through the 1970s. In the early 1900s, carbon disulfide was marketed for control of bots and ascarids. Other compound classes were released between the 1940s and 1990s, including: piperazines, benzimidazoles, organophosphates,

pyrantels, and macrocyclic lactones. At present there are four classes of compounds on the market: benzimidazoles, macrocyclic lactones, piperazines, and pyrimidines (Lyons et al., 1999). Each class has it owns strengths, weaknesses, and mechanism of action.

Cyathostomes are the most prevalent nematode parasites of horses worldwide. Also known as small strongyles, cyathostomins, or trichonemes, these nematodes fall under the Superfamily *Strongyloidea* and include over 50 species and several genera. The adults of the different species can be identified due to morphological differences, but the ova and larvae cannot. The eggs are morphologically identical, thus identifying them under a microscope is impossible (Andersen et al., 2013). For the purpose of this study specific genera and species are not relevant so "cyathostome" will be used generically throughout.

Cyathostomes are the most important parasite of mature horses. They gained this distinction as a result of three well-known reasons. First, large strongyles have been eradicated in well-managed farms. Second, other nematodes are found exclusively in juvenile horses because of immunity, *Parascaris equorum* being the greatest example, whereas cyathostomes can impact horses of all ages. Lastly, the remaining prevalent parasites in equines, such as bots and pinworms, are typically less pathogenic than cyathostomes.

It is important to understand the biology, pathogenicity, and epidemiology of parasites for an accurate diagnosis. Cyathostomes are ubiquitous, in that they can infect any grazing horse in essentially any climate or geographical conditions (Nielsen et al., 2010). Small strongyles alone have 51 identified species in 13 genera (Anderson et al.,

2013; Hodgekinson, 2006). Cyathostomes have a direct life cycle, meaning they only have one host, the horse. The eggs are passed through the feces, then develop into first, second, and third stage larvae on the ground. Prepatent development is usually between 6-12 weeks (can be years with arrested development in the early encysted L3 stage) (Hodgekinson, 2006). The third stage larvae are infective and will reenter the host through the mouth, where they develop into adults (Lyons et al., 1999). The adults live in the lumen of the large intestine and produce eggs. The preparasitic stages have temperature dependent development on pasture until they reach L3, which is ingested into the horse. They shed their sheath and encyst in the large intestinal mucosa. Small strongyles have only had a general life cycle described. Because there are so many species, there has only been scattered information put together.

Small strongyles are generally less pathogenic but encysted fourth or fifth stage larvae can emerge in mass from the mucosa of the large intestine. This is known as cyathostominosis. In extreme cases, this can rupture mucosal cells and cause severe loss of fluids and protein that can lead to death. Despite the potential danger, virtually 100% of horses are infected with small strongyles to some extent. The total number within a host ranges depending on several factors including age and climate conditions. And while the number of species of small strongyles is in the 50s, only about 10 species make up the bulk found in horses. Some of the more rare species only show up in donkeys or zebras (Lyons et al., 1999). They present with several clinical issues, but when large inactive encysted larvae activate at the same time it can cause severe inflammatory colitis. It proves difficult to manage this because anthelmintics cannot treat early

encysted larvae and resistance against these drugs is increasing (Hodgekinson, 2006). Moxidectin, a member of the macrocyclic lactones, is the only anthelmintic effective against the late stage L3 encysted larvae. While this drug helps prevent mass emergence it does nothing for the larger population of early encysted L3s.

Resistance:

Resistance is a heritable genetic change in a parasite population that enables a greater proportion of individual parasites to survive anthelmintic treatments that were previously effective, assuming the populations are of the same species and developmental stage. Resistance is very dynamic and changes occur slowly over time as the "resistant" alleles begin to increase in frequency. There are many factors that affect the development of resistance, such as the mode of inheritance, the number of genes involved, the specific parasite biology and epidemiology, the timing and frequency of drug treatments, and the pharmacokinetics of the drug. Because of this slow development, resistance is not noticed until the alleles are in high numbers. In a highly pathogenic parasite, the failure of therapeutic treatment can be the manifestation of resistance but parasites like cyathostomins with low pathogenic potential resistance can only be detected if testing is performed (Vidyashankar et al., 2012).

Resistance against anthelmintics has been reported widely in *P.equorum* and cyathostomes, and the equine industry has begun to take steps in developing a more selective approach to controlling internal parasites (Andersen et al., 2013).

Avermectin/milbemycin resistance is reported in sheep and cattle, and in equine roundworms. Strongyle populations are widely resistant to pyrantel salts and

benzimidazoles (Nielsen 2010). Ivermectin remains 99% effective. Moxidectin is 99% effective and more than 60% effective against encysted larval forms (Molento, 2012).

In a 2010 study using sheep, it was shown that when benzimidazole resistance is 25%, the medium level, in a population no reversion is possible even when the anthelmintic is switched. The refugia, or remaining parasites not impacted by treatment is not enough to allow genetic drift to occur (Leignel, 2010).

There have been multiple studies used to determine if drugs remain effective against parasites, but many of them are biased or use improper statistical evaluation. A study done in 2001 showed that moxidectin and ivermectin were still the gold standard in deworming and suggested that horses should be dewormed more frequently with moxidectin in order to keep parasite populations down (Martin-Downum, 2001). This conclusion is contradictory to most equine parasitologists' recommendations around the world. Instead, it is important to maintain the efficacy of the drugs on the market since it is unknown when a new drug class will become available. Current drug classes should be treated as valuable resources. The current calendar-based strategy takes no consideration to the size or species makeup of the parasite burden at different times of the year or resistance status. So, horses end up receiving unnecessary and excessive treatments (Nielson et al., 2007).

Horse Behavior and Natural Refugia:

Pastures grazed by herbivores contain mosaics of grasses in different patches made up of different physical structures and compositions. Optimal forage models use only nutrition as the primary goal but there are other factors, such as risk of parasitism

that should be incorporation into the model for a more accurate picture. Parasites impact the host's productivity through food utilization and absorption. As a result there is major selection pressure by the host to reduce the risk of encountering infective stages and reduce the negative impacts of infection. As the eggs pass onto the vegetation they develop into the infective L3 stage. The horse cannot detect L3 but they have been shown to avoid feces as a mechanism of "avoiding" egg exposure. In sheep studies, the parasitized animals avoid the feces even more than the unparasitized animal (Hutchings et al., 1998). This difference has not been identified in horses. Horses tend to create and maintain short patches of grass and concentrate their feeding time there, with a preference for immature vegetation. These shorter patches are often within a matrix of patches of taller grasses and feces. A study conducted in France by Fleurance (2007) suggested horses avoided short patches near fecal piles, as well as tall grasses (feces or no feces). This has nutritional influence as well because tall grass is generally lower quality (Fleurance et al., 2007).

The Fleurance study also focused on the climate factors that effect refugia and how that relates to parasite control strategies. Parasites not being exposed to the drugs during treatment are considered "in refugia" because they are not under selection pressure for resistance. Part of the reason horse owners have been recently encouraged not to over-treat their horses with anthelmintics is because it reduces the number of parasites in refugia (Nielsen, 2012). This includes free-living stages, parasites in untreated horses, and the encysted cyathostomes. These parasites provide a source of genetic material to dilute the resistant alleles. This has been confirmed with sheep studies. So, to reduce

selection pressure, one should treat horses at times when pasture refugia is high and reduce treatment when refugia is low (Fleurance et al., 2007).

Weather and Seasonality:

In laboratory studies, alternations of thaw and frost are harmful to most stages of strongyles. L3 stages can survive long-term storage at alternating temperatures but often can no longer infect the horse.

In the field studies, northern climates have uniform rainfall, distinct seasons, cold winters. Rapid parasite development occurs during late spring, summer, and early autumn. Winter has slow and impaired development of egg and larval stages. The larvae however can persist for several months and when the temperatures warm up, L3 will persist as the intact fecal balls serve as protection. This suggests that disrupting the fecal ball can expose the parasite to the climate and kill them. Intact fecal balls act as reservoirs and intermittently release L3 on the pasture (Nielsen et al., 2007). This often results in the management recommendation to spread or harrow fecal piles in pasture when possible to break up fecal balls and expose any eggs.

Warm and hot climates have less distinct seasons, infrequent frost, and summers are consistently hot. Larvae have been shown to be negatively impacted by hot weather, regardless of moisture. If wet, larvae rarely survive. While if dry, larvae are trapped in the fecal ball and cannot migrate. The life span is thus shortened. During a drought, the larvae is not seen on the pasture, but as soon as there is moisture, the larvae return (again with the fecal ball as a reservoir). The highest amounts of strongyle numbers occur in autumn, winter, and spring.

Treatment should match appropriate refugia. In cold climates: spring/summer/early fall. Hot climates: fall/winter/spring (Nielsen et al., 2007).

Evidence-Based Control:

There are two categories of control: chemotherapy and management. Chemotherapy is treatments of parasites using drugs, while management refers to controlling parasite populations in the environment. Management practices include: rotation of pastures, alternating ruminants and horses on pastures, harrowing pastures, and using natural sources, such as dung beetles, who bury the feces and destroy the parasites. Frequently people will try to eliminate all parasites from an equine population but doing so is impractical and can harm the environment by exposing it to so many chemicals so frequently (Lyons et al., 1999).

In the 1960s treatment programs were focused on large strongyles, and horses were dewormed on an interval program of every eight weeks to prevent parasite transmission. However, large strongyles are almost eradicated and the problem now is with cyathostomes (Nielsen, 2012). Fecal egg counts (FEC) for selected therapy developed in 1991, first in cattle and sheep. FECs are also used with domestic animals like dogs and cats. It is a method for determining the number of nematode eggs per gram of feces in order to estimate the worm burden in an animal. They are used for diagnosis and surveillance. The fecal egg count reduction test (FECRT) is considered the gold standard since 2002 (Andersen et al., 2013). The FECRT documents the resistance of a parasite in a certain group to a certain anthelmintic. The population being tests receives a baseline FEC and then is treated. After the appropriate number of days (differs

depending on the drug used), a second FEC is performed. The percent FECR is (FECpre - FECpost / FECpre) x 100.

Utilizing the FEC or FECRT, the goal is to reduce treatment intensity and delay the development of resistance by analyzing fecal samples from each individual horse and only treating the horses that show above a pre-determined value of eggs per gram. It is also recommended a farm performs FECRT to monitor the development of resistance on the individual farm (Nielsen et al., 2010). With resistance looming, it is important to remember that the goal of modern parasite treatment is to treat just enough. There are risks in under- and over-treating horses for parasites. The hardest part of parasite research is ensuring that the horse owner receives and understands the appropriate message (Nielsen, 2012), thus owner education is key.

Danish perspective:

In 1999, anthelmintics became prescription-only in Denmark, putting the control of parasites in the hands of veterinarians instead of the horse owner (Andersen et al., 2013).

A survey was completed by 87 Danish Veterinary clinics on their practices regarding strategies for surveillance and control of parasites. The research showed that practitioners play a central role, as opposed to in the U.S. where the horse owner controls deworming decisions with little help from a qualified individual. The Danish vets used mostly FEC and larval cultures in diagnosing parasites. Cyathostomes were considered the most important parasite, and large strongyles was second (41% used larval cultures to detect *vulgaris*). Larval cultures were correlated to the caseload of the practice, which

was explained as laboratory availability and amount of technicians available. *Vulgaris* is a concern among Danish vets because it is prevalent at up to 20% at the herd level, according to recent reports. This is thought to be because of lower treatment intensity (Nielson et al., 2006).

Tapeworms appeared not to be a priority, which could be because they are hard to detect with FEC or simply out of a lack of concern. Also, an assay is more expensive because it requires the vet to take a blood sample.

Practitioners reported suggesting two fecal samples per year, at the beginning and end of the grazing period. 95% admitted to treating horses without fecal analysis, usually foals and young horses, and horses with signs of parasitic disease…this is thought to be the case because clinical disease is caused by the larval stage. Horses under 3 years old were found to be treated several times during a grazing season. Macrocyclic lactones (ivermectin, moxidectin) were identified as the most commonly used drug class, though 43% recommended the cheapest drug (Nielson et al., 2006).

Parasitologists remain concerned about resistance to moxidectin because it has long-term efficacy and diminished parasite refugia in this study. Pyrantel was also identified to have been used, even though there are Danish reports of resistance. While it is the responsibility of the practitioner to prescribe the best anthelmintic, the survey shows they often rely on the efficacy of ivermectin and moxidectin instead of performing FECRT. It was also noted that horse owners illegally import anthelmintic drugs from out of the country (Nielson et al., 2006).

As a follow up to these regulations, a study was done in 2012 measuring the

reemergence of large strongyles on Danish farms (Nielsen et al., 2012). Surveying 42 farms across the country and a total of 663 horses of various types and uses, the authors performed FEC and larval cultures throughout the three-month study. They concluded that large strongyles were present and detectable across the country, but to be sure it was caused by the prescription-only anthelmintics, they would need to do a more thorough examination. One of the theories is that the reemergence is because some of the horses have not been treated in several years as they have not shown clinical signs of infection (Nielsen et al., 2012).

FEC Problems and Factors of the Test:

FEC only indicate the presence of adult worms in the intestinal lumen. It takes no account of the encysted or migrating larval stages, which are the pathogenic stages. This means the diagnosis for large and small strongyles occurs after the damage potentially occurs (patency). Prepatency is the time from when the parasite enters the host until it reaches sexual maturity. While the prepatent stage is shortest for small strongyles, they can arrest development and remain encysted inside the lumen of the large intestine for years (Andersen et al., 2013).

FECRT is the most widely used *in vivo* method of assessing efficacy of anthelmintics in horses, sheep, and cattle. Its limitations include: variability of FEC data (resulting in inconsistent FECRT), age related immunities, gazing management (greater variability with horses than sheep or cattle). Because FECRT are the gold standard, it is urgent to improve and standardize the statistical methods of analyzing the data (Denwood et al., 2010). The traditional diagnosis is FEC, but horses with cyathostominosis often

have egg counts of zero. This is because it is caused by the larvae and not the adult stages.

Because fecal egg counts are the gold standard, a study was conducted to measure the collection and storage factors on strongylid eggs (Nielsen et al., 2010). The investigators collected FEC on horses testing in the 200-500 EPG range, identified as having enough eggs to observe in the two different countries with two different climates. The locations included summer in Athens, GA, USA, and autumn in Copenhagen, Denmark. They completed two objectives. The first objective looked at temperature and storage time. Each fecal sample was placed in a freezer bag and put in a freezer, a refrigerator, an incubator, or kept at room temperature. The egg counts decreased in the freezer by 24 hours. There were no decreasing numbers in the refrigerator. Room temperature results differed by country. In Denmark there was a trend for decreasing numbers, but in the Georgia the FEC remained stable over time. There was also a trend for decreasing numbers with the samples kept in the incubator. The second objective measured the difference between fecal samples immediately put into airtight containers and samples left exposed to open air. They measured the FEC over a 24h period. The Danish study saw no difference, but the Georgia study showed a significant decrease in FEC in the open air containers, especially during the first 12h. This study confirms that storage factors will affect the accuracy of the FEC and should be taken into consideration in an evidence-based control program. Samples should be immediately placed in an airtight container (a sealed bag is the common choice) and should be stored at refrigerated temperatures. The differences observed in the studies, especially referring to

the room temperature storage were explained by the common presence of air conditioning in the United States, as it is rare in Denmark. The overall message of this study is the eggs can be collected from the ground for FEC within 12h and can be stored for up to 120h in a refrigerator (Nielsen et al., 2010).

Diagnostics:

Larval cultures performed on fecal samples can identify the parasite as a cyathostome, but it cannot distinguish between species. Two other diagnostic tests exist: a species specific PCR-ELISA (enzyme-linked immunosorbent assay) and RLB (reverse line blot) assays. These are much faster than larval determination via morphology and they can recognize up to 21 cyathostome species (Andersen et al., 2013). However, these tests require DNA from a sample, usually taken from a fecal sample, which is also only reflective of the mature parasites in the intestinal lumen. So it does not quantitatively reflect the total parasite burden. Recognizing emerging L4 is also a challenge morphologically, but it is important because L4 is the stage responsible for cyathostominosis. There are two antigen complex diagnostic tests are being developed to diagnosis this stage using antigen specific IgG(T) (Andersen et al., 2013). Antibodies increase 5-6 weeks after infection (Andersen et al., 2013). The same lab has further discovered cyathostomin-gut associated larval antigen 1, which is only expressed during larval stages. The ELISA development of this test still needs work to be quantitative, but it would be the first prepatent diagnostic assay for developing small strongyles.

Historically, a single technique has been used to enumerate the two mucosal populations (EL3s and DLs). Both can be liberated from the tissue by digestion in a

solution of pepsin + hydrochloric acid, and enumerated microscopically. The DL stages occur superficially in the gut mucosa, and may not be encysted (*i.e.*, surrounded by a layer of fibrous, scar tissue of host origin), so extensive digestion shouldn't be required to liberate them for quantification. On the other hand, far more digestion is required to release the EL3 stages, but it is uncertain what percentages of either category are lost in the process.

Because of the size and anatomic site differences among the various life cycle stages, different techniques have been used historically to quantify the respective populations. Due to the very small size of EL3s, they cannot be observed or counted *in situ*. Historically, EL3 numbers have been determined by digesting weighed samples of mucosa in pepsin and hydrochloric acid, and counting the liberated larvae microscopically. The larger stages (LL3s and EL4s) can be viewed and counted *in situ*. It is believed that they do not survive the digestion process very well, so their numbers are determined by direct observation and manual counting of the stages visible in representative samples of the gut mucosa.

It is important to be able to accurately quantify the various portions of a larval cyathostomin population. Efficacy of different drugs (*i.e.,* larvicidal anthelmintics) against the various subpopulations can only be determined by calculating the differences in worm numbers between treated and untreated horses. Furthermore, the biology and seasonal transmission of these parasites could be investigated better by accurate assessment of quantitative changes over time. The original methods for larval quantitation were published over 30 years ago (Eysker, 1984; Reinemeyer et al., 1986),

identifying the need for further study in this area.

Because FEC can only account for the adult worms in the lumen and most anthelmintics can only treat those same worms, there is a growing need to research the encysted larval stages in the mucosa of the large intestine. EL3s, LL3s, and L4s are the most dangerous stages of cyathostome in the horse. Researchers developed a larval quantification technique over 30 years ago to be able to study these parasites over time, as well as look at drug efficacy, seasonality, and biology of the nematode. The test recommended that weighed samples of mucosa are digested in pepsin and hydrochloric acid, and then the liberated larvae are counted microscopically. The digestion is traditionally allowed to proceed for a period of 2 h. However, this test was never fully optimized. The objective of this study herein then is to evaluate the time interval for optimal digestion in order to liberate the most larval stages possible from the tissue without damaging the larvae. We hypothesize the EL3 stages will require more time for digestion, while the LL3 and L4 stages will begin to become damaged the longer they are kept in the pepsin hydrochloric acid solution. Further, we hypothesize the optimal digestion times will differ for each stage, being longer for the EL3s and shorter for the LL3 and L4 stages (collectively known as developing larvae or DLs). The results of this research will provide researchers another tool for accurately measuring the encysted parasite load of the horse, effectively increasing the accuracy of drug efficacy studies, as well as observing parasite seasonality and biology, and thus may impact the quality of life for horses worldwide.

METHODS AND MATERIALS

This study was approved by the Institutional Animal Care and Use Committee at East Tennessee Clinical Research, Inc. Five horses, identified as likely to have burdens of larval small strongyles via FEC, were humanely euthanized by a licensed veterinarian. The cecum and ventral colon were removed using traditional necropsy techniques within 1 h of death.

Mucosa was harvested from the cecum and ventral colon using the long edge of stacked, glass microscope slides to scrape the mucosa from the underlying serosal tissues. The serosa was discarded. The entire collection from each organ, processed separately, was chopped using the edges of the slides and transferred into a labeled container until >300 grams of tissue were recovered from each organ. Tissue was immediately transported to the nearby laboratory for analyses.

Twenty-five 10-gram aliquots of mucosa from each organ were processed on the day of collection. An additional five aliquots from each organ was prepared and placed into frozen storage.

Ten grams of scraped mucosa was transferred to each labeled, 500mL Erlenmeyer flask. To each flask of scraped mucosa, ~100mL of digestion solution was added. Digestion solution was prepared beforehand by dissolving 10g pepsin (1:10,000) in 1 liter of warm water, and adding 15mL of concentrated hydrochloric acid. Each Erlenmeyer flask was capped tightly and agitated at approximately 37ºC for the appropriate time interval. "In" and "out" times were recorded. The five replicates of tissue were digested for four different time treatments: 60 min, 90 min, 120 min, and 150 min for each organ.

Upon expiration of the respective time interval, the contents of the flask were

poured into 2 L tubs and diluted by pouring the contents up to 1,000 mL with tap water. After thorough mixing, a 25% representative aliquot (5 x 50mL samples) of the digest was collected and washed vigorously over two stacked sieves, #8 sieve and #230 sieve with apertures of 63 um. The #8 sieve trapped large particles that were discarded. The contents trapped in the #230 sieve were washed into a petri dish with a tap water squirt bottle and stained with Lugol's iodine. Nematodes absorbed the iodine stain and subsequently appear as red or brown against a clear background.

The stained digest sample was examined microscopically at a 15-30x magnification by trained examiners. Early third stage (EL3) cyathostomin larvae were counted and recorded as numbers of EL3 on a specific date capture form (DCF). Late third stage (LL3) and fourth stage (L4) larvae present are counted separately from EL3s and recorded as numbers of developing larvae (DL) on a specific DCF. EL3 cyathostomins are identified as being <1mm, “c” or comma shaped, and having no oral cavity. LL3 cyathostomins appear larger than EL3 and have a tubular oral cavity. L4 are large and have a rectangular or non-tubular oral cavity. No distinction was made between LL3 and L4 in this study; both were counted together and a single number per sample was recorded.

Data were tested for normality using the Shapiro-Wilk statistic. A mixed model with repeated measures was used to examine effects of digestion time on cecum and ventral colon locations independently, with larvae count as the dependent variable, horse as the subject, and time by replication as the repeated effect using SAS 9.2 (Sas Inst, Cary, NC). The Curvilinear relationships between larvae in the cecum vs ventral colon

was examined and fit using graphical analysis software (SlideWrite Plus Ver. 7, Advanced Graphics Software, Inc., Rancho Santa Fe, CA).

RESULTS

EL3:

The number of EL3s increased from time interval 60-150 min in the cecum (Figure 1). The numbers of EL3 also exhibited a time effect ($p = 0.035$), increasing in the ventral colon from 60-90 min, remaining unchanged from 90 min at 120 min, and showing a further increase to 150 min (Figure 2).

Early stage L3: Cecum

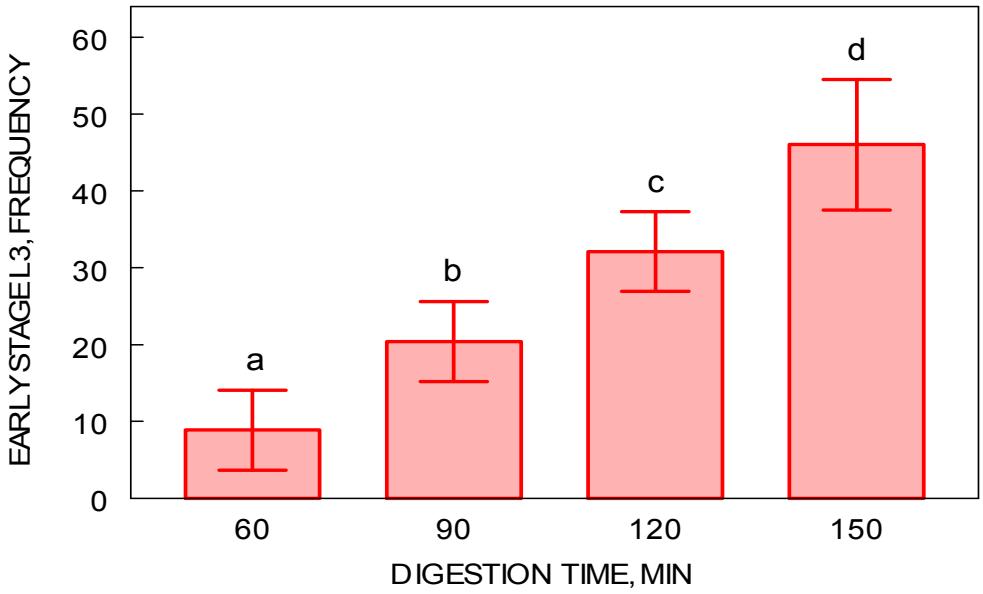

Figure 1- Mean numbers of cyathostome EL3 liberated from equine cecal mucosa and digested from 60 to 150 min in a pepsin/hydrochloric acid solution. Time points not sharing a similar subscript are different ($p = 0.032$).

Early stage L3: Ventral Colon

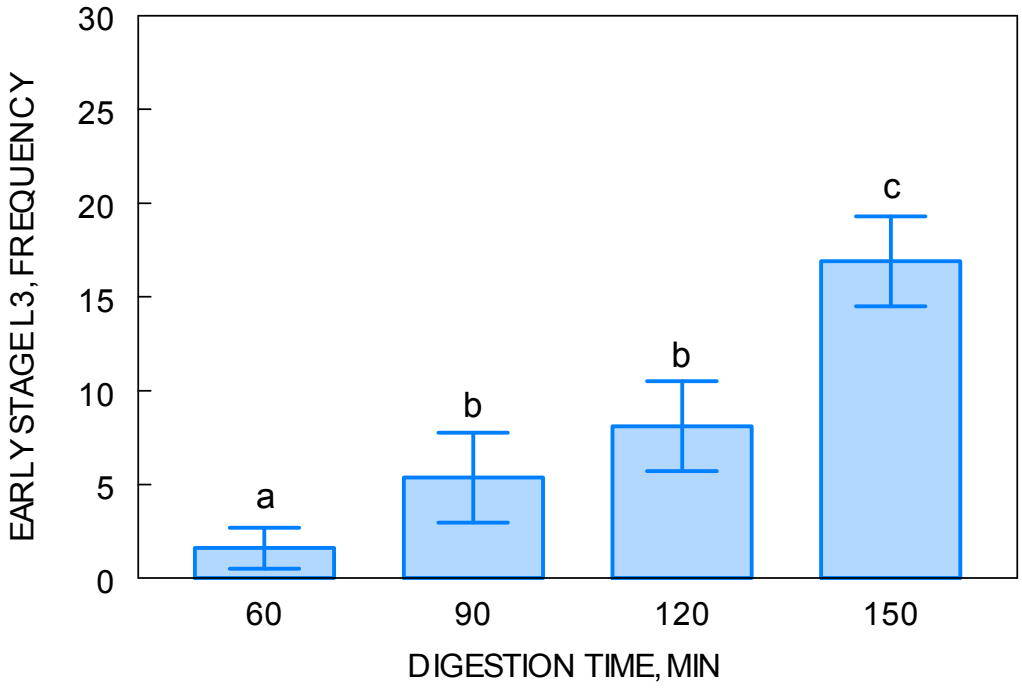

Figure 2- Mean numbers of cyathostome EL3 liberated from equine ventral colon mucosa and digested from 60 to 150 min in a pepsin/hydrochloric acid solution. Time points not sharing a similar subscript are different ($p = 0.035$).

DL:

There was no difference in the numbers of DLs present from time intervals 60-150 in either the cecum ($p = 0.71$; Figure 3) or the ventral colon ($p > 0.67$; Figure 4).

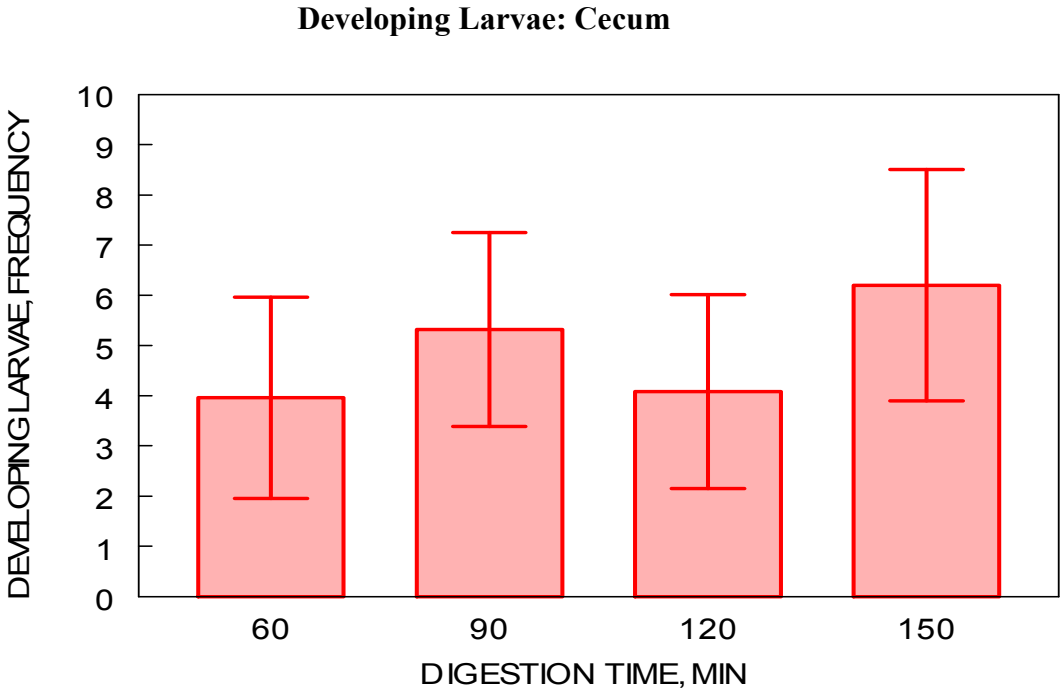

Figure 3- Mean numbers of cyathostome DL liberated from equine cecal mucosa and digested from 60 to 150 min in a pepsin/hydrochloric acid solution.

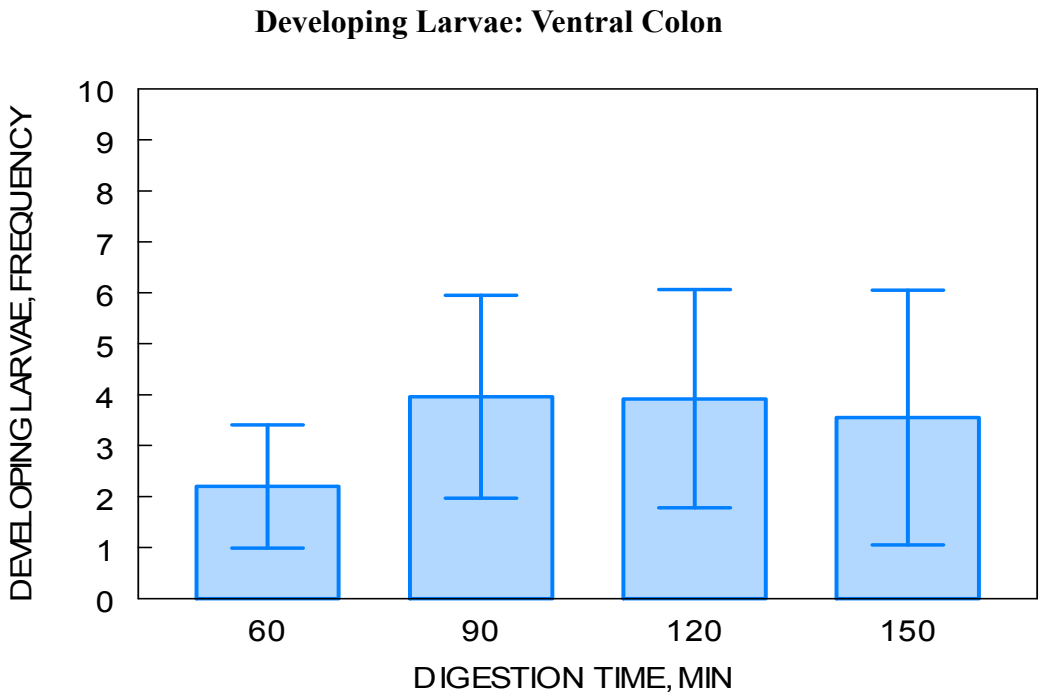

Figure 4- Mean numbers of cyathostome DL liberated from equine ventral colon mucosa and digested from 60 to 150 min in a pepsin/hydrochloric acid solution.

Ventral Colon vs Cecum:

Presence of larvae in the cecum and ventral colon were positively correlated (R = 0.82; $p < 0.0001$; Figure 5), where as an increase in larval count in the ventral colon was correlated to an increased larval count in the cecum.

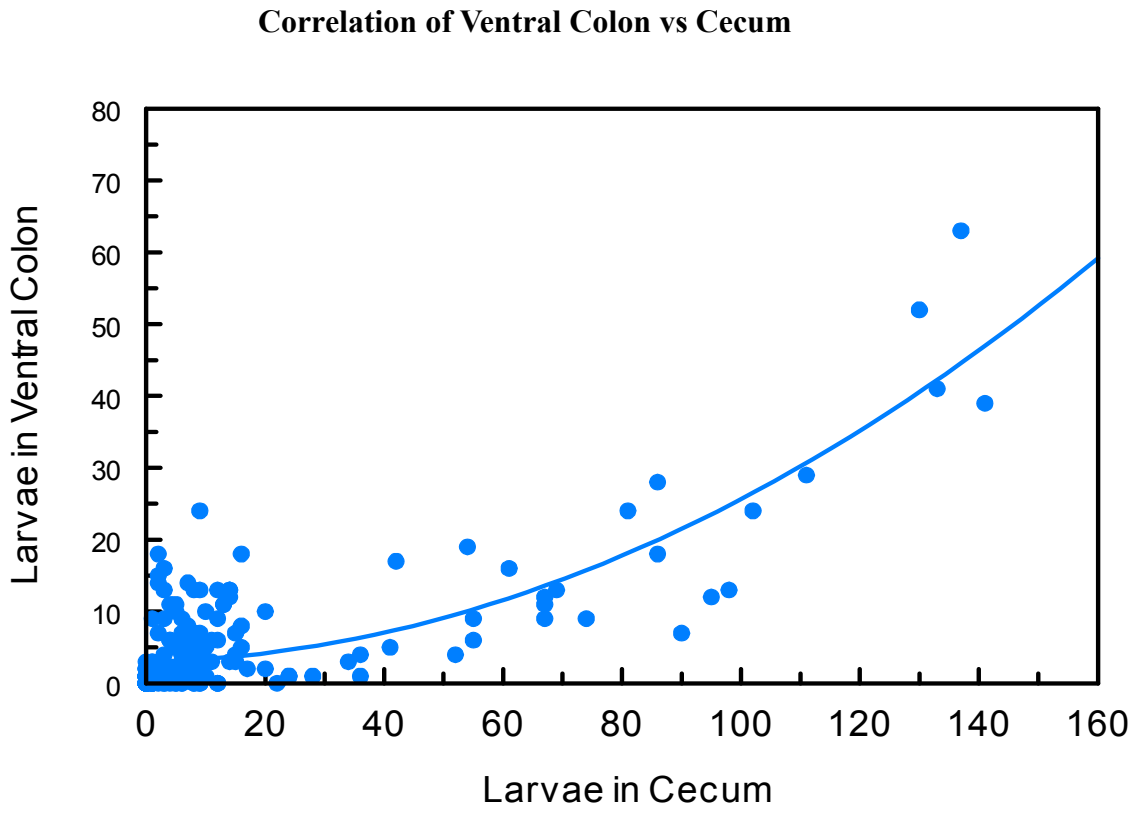

Figure 5- A correlation graphed between larvae found in the ventral colon and larvae found in the cecum, indicating if larvae are found in one organ, they are found in the other organ.

DISCUSSION

The numbers of EL3s found was greatest at the 150 time point. This shows that the longer the tissue samples are digested, within the time frames of this experiment, the more EL3s are released from the mucosa. The standard for this enumeration technique is 120 min, but this study suggests that there was a difference between 120 and 150 min ($p = 0.001$). In future studies, longer time intervals would be needed to reveal the optimum time for liberating EL3 larvae from the tissue. The cecum contains more of the parasite burden than the ventral colon, so the increased larval counts in the cecum compared to the ventral colon were expected.

The numbers of DL found was not different between each time interval. This shows that no matter how the long the tissue sample was digested, there was no real change between the DLs present. However, while counting the plates, the technicians (Emily Fitzgerald, Dr. Craig Reinemeyer, Abby Pritchard, and Josie Collins) observed that once the tissues were digested past the 120 min time interval, the DLs began to be broken down by the digestion process. The borders of the larvae became less clear and the insides began breaking apart. The technicians counted the larvae as present, despite the loss in quality, so the numbers do not reflect a change. In future studies looking at this technique, the degrading DLs should be counted separately and the level of disintegration should be noted, aiding in identifying the time at which digestion has gone too far for the DLs.

Based on these results, it is clear that this enumeration technique is not adequate for comparing EL3s and DLs at the same time. The Mural Transillumination Technique

(MMT) was developed by Dr. Craig Reinemeyer 30 years ago as a way to enumerate DLs without digestion. As DLs are visible to the naked eye, and even more so magnified microscopically, when he took a piece of gut wall and placed it mucosa side up in a petri dish, he could count the numbers of encysted larvae with the aid of a superimposed grid. A modified version of the technique would involve taking the tissue sample by weight and separating the mucosa from the thicker underlying tissues by sharp dissection (like filleting a fish). Looking through the mucosal layer alone would vastly improve the visibility and lead to a more accurate count. This modified MMT could then be compared to samples that undergo digestion and the differences could be measured. Perhaps then we would have a separate test for DLs that was just as accurate as the digestion procedure is for EL3s.

In conclusion, the mechanical digestion technique is a commonly used and respected laboratory procedure used by parasitologists world-wide to enumerate encysted stages of cyathostome nematodes in the horse. It is clear from this study that the technique needs to be examined more fully to truly optimize its potential as an accurate test. With the optimization of mechanical digestion, more accurate studies on drug efficacy and studies of cyathostome biology can be performed. With more accuracy in future studies, research can make greater strides in looking at issues such as resistance, developing new drug classes, and preventing cyathostominosis.

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APPENDICES

APPENDIX A: IACUC APPROVAL

RE: ETCR-14-0143

Julio Prado

Fri 10/17/2014 2:33 PM

Inbox

To:Craig Reinemeyer <creinemeyer@easttenncr.com>; Bree Reinemeyer <breinemeyer@easttenncr.com>; Maria Prado <mprado@utk.edu>; Misty Nelson <mnnelson@hotmail.com>; Michael M Fry <mfry@utk.edu>; Misty Nelson <mnnelson@roaneschools.com>;

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Dear Dr. Reinemeyer and fellow IACUC members,

The IACUC committee members were in agreement that a designated review was adequate for protocol **ETCR-14-0143: "Optimization of techniques for quantifying encysted cyathostomin populations in the cecal and colonic mucosa of horses.".** As chair of this committee I appointed **Ms. Bree Reinemeyer** as the designated reviewer, **She** thoroughly reviewed the protocol and any questions that **She** had were addressed by an ETCR member.

The protocol **ETCR-14-0143** submitted for review to the ETCR IACUC committee does not present any unresolved animal welfare issues and is **approved**.

Call me if you have any questions or comments.

Julio C. Prado, DVM
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