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Resistance of Abomasal Parasites to Common Anthelmintics in Small Ruminants in the Northeast

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RESISTANCE OF ABOMASAL PARASITES TO COMMON ANTHELMINTICS IN SMALL RUMINANTS IN THE NORTHEAST

by

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ABSTRACT

The abomasal worm of ruminants, *Haemonchus contortus,* is detrimental to Northeast sheep and goats. It is also often resistant to anthelmintics commonly used by sheep producers in Maine. We developed methods to assess response of these parasites to anthelmintics. Fecal samples from experimentally infected sheep in West Virginia and from two farms in Maine were used as sources of parasite ova and larvae in these experiments. Parasites were grown to the L3 stage, examined, and then studied using motility tests, Methylthiazol Tetrazolium (MTT) assays and spectrophotometry. Due to selection for resistance over time in Maine parasites, we expect anthelmintics to have a higher kill rate on parasites obtained from West Virginia than on parasites collected from Maine.

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INTRODUCTION

Parasitism, and abomasal parasitism in particular, is one of the most serious constraints affecting small ruminant production in the Northeast. Abomasal parasites are detrimental to the health of the individual animal and are very difficult to eliminate from a herd or flock. The incidence of parasitism in the Northeast has been rising in recent years, and this might be partially due to overuse of anthelmintics in commercial sheep and goat farms, which could be allowing the parasites to adapt and become resistant to these anthelmintic drugs.

Haemonchus contortus is the most pathogenic of the different species of intestinal parasites. It is an aggressive parasite that survives by feeding on the blood of the sheep or goat while in its adult stage and living in the abomasums of the animal. An infection by this parasite can easily cause anemia and other health complications, which can be fatal if left undiagnosed and untreated.

For this project, experiments were designed to identify which anthelmintics provide at least a 50% kill rate on the parasites at varying larval stages. The experiments involved using laboratory techniques to identify the number of parasites in each sample, and then adding the commonly used anthelmintics to the samples to determine the most effective products. A project such as this had never been undertaken at this facility before, and our project focused mainly on validating parasite identification techniques in our lab that were already reported in the literature. We also attempted to develop a less expensive and simplified version of an Australian parasite antihelmintic resistance test (DrenchRite Assay).

This project provided valuable information to the University of Maine Diagnostic lab and set up a base of knowledge that will act as a foundation for future projects. We ran into a few problems that changed the focus of our project, but we learned a great deal that may be used to advance further research.

In most small ruminant production areas, gastro-intestinal parasites are the most economically important disease of sheep and goats. Small ruminants are more susceptible to and are affected more harshly by abomasal parasites than most other types of farm livestock. Small ruminant producers rely heavily on anthelmintics to control internal parasites in their flocks, but the long-time use and overuse of these drugs has resulted in parasites that have become increasingly resistant to anthelmintics. The development of anthelmintic resistance poses a threat to future production and welfare of small ruminants. It is important to determine the resistance of the abomasal worms in order to develop a strategy for controlling the parasites and delaying the development of resistance (Papadopoulos, 2008).

The development of resistance to all of the major groups of anthelmintic drugs has been found in many different intestinal parasitic species. Papadopoulos hypothesized that a small number of worms survive the anthelmintic treatments administered by the farmers, and these surviving worms are the strongest and most resistant of the population. These worms then contaminate the pasture with a majority of resistant eggs for the later generations, leading to the increased resistance of the entire species (Papadopoulos, 2008).

Álvarez-Sánchez (2006) found similar indications of resistance when he performed a study on the prevalence of parasite resistance on local farms in Spain. Three

anthelmintic drug families were surveyed, and resistance to all three of the anthelmintics was found. It is important to be able to detect the amount of anthelmintic resistance in a herd or flock because resistance can develop slowly at first and then escalate relatively quickly, making an entire population resistant in a short amount of time.

The first difficulty in determining the level of anthelmintic resistance is finding a way to identify how well an anthelmintic drug works on a parasitic population. An assay to determine how well the anthelmintics work by comparing the motility of the parasites before and after the anthelmintics were administered is a suitable method to determine the level of resistance that has evolved in certain species of parasites (Smout et al., 2010).

It is important to have a method to quickly and easily determine the viability of the different species of abomasal worms in order to develop new anthelmintics for use. Peak also praised the use of dyes or stains to identify the live-to-dead ratio of different parasitic species before and after the anthelmintics were administered (Peak et al., 2011).

Objective monitoring of parasite resistance in ruminants, pigs, and horses was determined as the first step in the development of new methods of parasite control (Coles et al., 2006). Several different tests were performed on the abomasal parasites in order to determine resistance, such as fecal egg count and testing third stage larvae (Coles et al., 2006). Both techniques were used in our studies.

The 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used to determine the live-to-dead ratio of parasites in order to deduce a kill rate for each anthelmintic. MTT is a yellow, water soluble, tetrazolium salt. The dye is converted to water-insoluble purple formazan by the succinate dehydrogenase system of active mitochondria. Therefore, the amount of purple formazan formed could

be determined with the use of a spectrophotometer and serve as an indicator of the number of working mitochondria, and therefore the number of living parasites in the sample (Aziz, 2007).

Studies on the resistance of abomasal parasites to different anthelmintics have been performed in recent years. While none of the studies were completed in the Northeastern United States, similar studies in other parts of the world focused on different parasites. All of the studies determined that there is some level of resistance in every parasite species that was tested. This study formed a basis of knowledge more specific to our area than previous studies that were performed in other parts of the world that will allow future researchers to explore the parasite issue locally.

OBJECTIVES

1) To attempt to replicate the laboratory parasite identification and anthelmintic resistance techniques reported by other facilities.

2) To determine the viability of the *Haemonchus contortus* parasites that came from both the West Virginia experimentally infected sheep and the two sheep farms in Maine, and identify the anthelmintics that have at least a 50% kill rate on the parasites.

HYPOTHESIS

We expected that the *Haemonchus contortus* parasites that were shipped to us from West Virginia would be less resistant to the anthelmintics than the *Haemonchus contortus* parasites that were collected from the sheep farms in the state of Maine. We developed this hypothesis based on the fact that the parasites generated from the experimentally infected sheep in West Virginia had not come into contact with many anthelmintics, and had therefore not built up a level of resistance, while the parasites from the sheep in Maine had been treated with anthelmintics regularly.

MATERIALS AND METHODS

Fecal samples to be evaluated in this study were shipped from a facility in West Virginia that collected the samples from an experimentally infected sheep, and from two separate farms in the State of Maine. Each sample was be labeled accordingly with the location it came from and the details of its clinical case (if applicable).

For each fecal sample, feces were collected from the ground or from a "diaper" that had been affixed to the animal overnight. A modified McMaster test was performed on the feces to determine the eggs per gram (EPG) using a McMaster slide. This provided an estimate of the quantity of eggs present in the fecal sample to determine whether sufficient eggs were present for conducting a larval development assay. If we determined that there were not sufficient ova in the sample to perform an assay, it was carefully discarded. To perform the McMaster's count, a fecal solution was made in a vial by combining 30 ml of saturated sodium chloride with 2g of feces. The feces were carefully broken apart and the solution was mixed by gentle inversion. Immediately following mixing, a sample of the fecal solution was pulled from the center of the vial using a pipette and inserted into the two chambers of the McMasters slide. The slide was then left to sit for 5 minutes before being examined under a light microscope. At 100X magnification, the number of eggs that fell within the grid of each chamber was counted and multiplied by 50 to determine the eggs per gram (EPG).

Since it is technically difficult to determine whether an anthelmintic has killed a parasite at the ova stage, this project focused on the third larval (L3) stage of the life cycle. Cultures of feces were prepared to allow for the development of L3 larvae. For each sample, approximately 5g of feces were broken into pieces and placed in the bottom of a plastic sandwich container. The sample was then mixed with equal parts of peat

moss and activated charcoal, and sprinkled with water to keep it from drying out. The peat moss was autoclaved before adding it to the fecal sample to eliminate any other competing bacteria or parasites that might have been living in the peat moss. The container was then covered with its lid and incubated at 26 degrees C for 7 days in a humidified incubator to allow parasite development to the L3 larval stage.

Upon completion of incubation, a Baermann Apparatus was set up in order to isolate the live parasites from the feces and other debris. This was accomplished by removing a portion of the cultures from the containers and placing it in the center of a Kimwipe paper cloth, with another Kimwipe placed on top to form a fecal packet. The fecal packet was then placed in the top of a glass funnel setting on top of a beaker, holding about 50ml of lukewarm tap water, with the stopcock in the closed position, making sure that the sides of the Kimwipe were up and away from the edge of the funnel to prevent capillary movement of fluid out of the funnel. The fecal packet was left for 3-5 hours to allow the L3 parasite larva to migrate from the fecal packet to the water and to settle in the bottom portion of the funnel. The Kimwipe has very small pores that allow the small parasite to actively pass through, leaving debris and dead parasites trapped in the packet. Larvae were then collected by opening the stopcock and letting the water with the parasites flow from the funnel into the beaker underneath. The remaining fecal packet was discarded.

The parasites were then prepared for long-term storage. The water with parasites that was just collected was transferred to 50 mL tubes and centrifuged to concentrate the parasites in a pellet at the bottom of the tube. The water was then removed and PBS

solution was added and the parasites were then stored in the refrigerator until needed. The concentration of each tube was determined to be 250 parasites per milliliter.

To remove the cuticle of the L3 stage larva, the parasites were washed with a diluted bleach solution. A 2% bleach solution was made adding 1mL of Clorox bleach (containing 6% sodium hypochlorite) to 50mL of water. This diluted bleach solution was then incubated with the parasites at room temperature. Parasite larvae were monitored under the dissection microscope for the shedding of cuticles, and once the parasites started shedding, one more minute was let pass before transferring the parasites to the rinse apparatus. The shedding process took about 15 minutes total. The parasites were rinsed using nylon sheeting in a set up similar to a Baermann. The nylon sheeting replaced the Kimwipe in this instance, and the solution was poured out onto the nylon sheeting sitting in the top of the glass funnel. The parasites were all caught in the small pores (30 micron openings) of the mesh while all of the bleach and liquid ran out through the funnel. A beaker was then placed under the funnel, the nylon mesh was carefully flipped upside down so the parasites were on the bottom of the mesh facing the glass funnel, and PBS solution was used to rinse the parasites into the beaker. The parasites, now without the cuticles, were then stored in PBS solution until needed.

The main test that was used in our experiments to determine whether the parasites were alive was the MTT assay, and controls were first set up to make sure that prolonged incubation of the parasites in MTT was not toxic. This was done by using four rows of five wells and adding a decreasing number of parasites in PBS to each well. To perform a 2x serial dilution, the first well in each row had 180 μL of the parasites in PBS solution added to it. Ninety μL of PBS was added to each well after the first well. Ninety μL was

then drawn up with the micropipet from the first well (containing the parasites) and added to the next well in the row, which already contained PBS. The solutions were mixed with the pipet and then 90 μL was immediately drawn up into the pipet and deposited in the next well. This was repeated down the row until the last well, which contained 180 μL instead of the 90 that each other well contained. This procedure was repeated for each row. The MTT assay was added next to the top two rows only, by adding 10 μL of MTT to each well, except the last well in each row which had 20 μL of MTT added to it. The bottom two rows had 10 μL of PBS only added to each well (except 20 μL to the last well in each row) in order to have a consistent volume throughout the plate. Every experimental well contained a final total of 100 μL, except for the last well on each row, which had 200 μL but the same ratio of solutions. Each well was then counted, and the number of parasites recorded, including noting any parasites that were not alive. The gradient of parasites should have been close to 25, 12, 6, 3, 1 based on this dilution strategy.

To test the control, the plate described above, with no anthelmintics added, was placed in the incubator at 27 degrees C for three days, and monitored for color change and movement of the parasites which indicated life.

Each successive 96-well plate that was made was created using the same method that was described above, unless indicated otherwise.

A 96-well plate was made for each of the three anthelmintics being tested. The three anthelmintics are: avermectin, benzimidizole, and imidothiazole. A plate was made for each of these three types of anthelmintics. Seven rows were used instead of four, the bottom three rows being control rows. The top six rows (rows A-F) were initially set up

as the control, with a set amount of worms in each well, while row 7 (row G) had no worms, only PBS. Ten μL of MTT assay was added to the top four rows. The three bottom control rows were used as three different controls. Row G had only PBS, MTT, and the anthelmintic with no parasites. Row F had parasites and PBS, but no anthelmintic or MTT. Row E had parasites, PBS, MTT, but no anthelmintic. This was done on each plate to ensure that the results were true and not due to a complication. Different amounts of anthelmintic were added to each row that required anthelmintic (all but rows E and F). In row A, 5 μL of anthelmintic was added to each well, except 10 μL was added to the last well. In row B, 10 μL of anthelmintic was added to each well, except 20 μL was added to the last well. In row C, 15 μL of anthelmintic was added to each well, except 30 μL was added to the last well. In row D, 20 μL of anthelmintic was added to each well, except 40 μL was added to the last well. In row G, the average, or 10 μL of anthelmintic was added to each well, except 20 μL was added to the last well. This setup can be seen in Figure A below.

Figure A: Set-up of a Typical 96-Well Plate in this Experiment

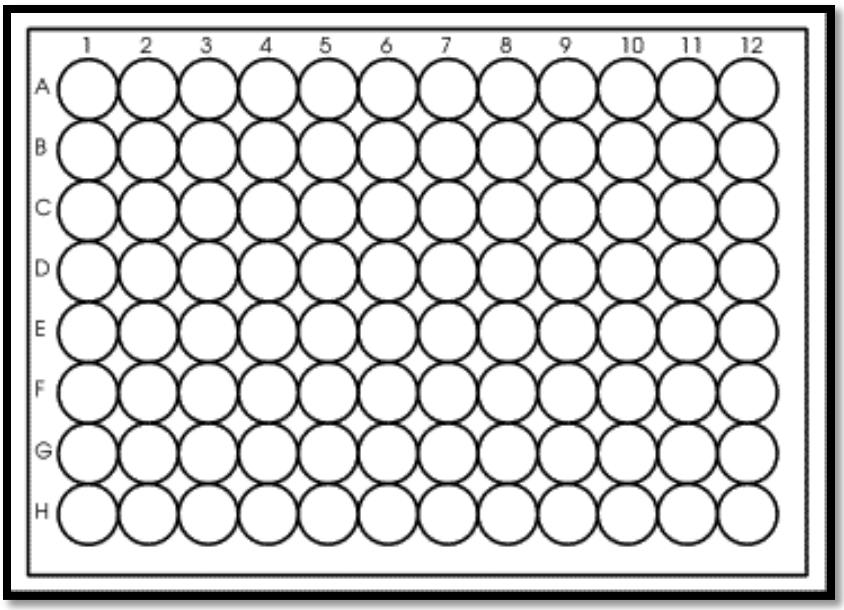


Table 1: Set-up of a Typical 96-Well Plate in this Experiment

Row A	Parasites in PBS, MTT Assay, and Anthelmintic
Row B	Parasites in PBS, MTT Assay, and Anthelmintic
Row C	Parasites in PBS, MTT Assay, and Anthelmintic
Row D	Parasites in PBS, MTT Assay, and Anthelmintic
Row E	Parasites in PBS, MTT Assay, and Anthelmintic
Row F	Parasites in PBS and MTT Assay (Positive Control 1)
Row G	Only Parasites in PBS (Positive Control 2)
Row H	MTT Assay and PBS, no parasites (Negative Control)

All of these plates were incubated for 48 hours at 27 degrees C in an atmosphere of 10% CO_2 in humidified air. After 48 hours, the plates were removed and examined for motility and life using the created rubric. All observations were made and noted, including if there were any discrepancies with the control rows. Acidified isopropanol was then added to each well containing MTT, and the wells were observed for a characteristic blue color change. If a color change was noted, plates were then read at 560 nm on a spectrophotometry plate reader to determine the level of absorbance. The rate of development of blue color in the solution was directly related to the metabolic activity of the L3 larvae. (i.e., if the solution remained fairly yellow, then there are few to no parasites still alive and metabolizing). Absorbencies were recorded, analyzed, and graphed to determine the level of resistance found.

RESULTS AND DISCUSSION

The anthelmintic potion of this experiment was unable to be completed, so the hypothesis was not proven or disproven. This experiment was started with very little previous knowledge of the techniques that were needed in order to grow, store, and test the *Haemonchus contortus* parasites. The first objective of this experiment was to research the laboratory techniques used by other facilities that have performed similar experiments and determine which techniques were replicable in our laboratory and would be effective when doing this type of research in the future. Many techniques were perfected and recorded through research and trial and error experiments.

Several problems were encountered with this project at different stages of the process. The first problem was the timing of the fecal collection. Unfortunately, many parasites, including the *H. contortus*, go into a hibernation stage and stop laying eggs in the winter months, which means that no eggs are shed in the feces and there were no eggs to be found when examining the feces in our initial collections. However, fecal samples were eventually collected that proved to be full of *H. contortus* from two farms in Maine and parasites were also ordered from a facility in West Virginia to complement the parasites that were found locally. Once the parasite infested fecal samples were obtained, the McMasters test was performed on each sample. It was determined that the McMasters test was a good indicator of the level of parasitic infestation in a given sample, and therefore in a given animal. It is a technique that will be continued to be used in the diagnostic laboratory at the University of Maine when giving initial information on a parasite infestation.

When initially attempting to grow the parasites, there was a problem with the parasites not surviving long enough to grow into the L3 larval stage. Upon closer examination, it was determined that there were other parasites and bacteria in the peat moss that were being unknowingly added to the culture, and those other organisms were outcompeting the *H. contortus* for nutrients and space. This problem was solved by autoclaving the peat moss before adding it to the culture.

The first 96-well plate that was made had a decreasing gradient (2x dilution from left to right across columns) of parasites in the wells. Four rows of 5 wells were used this first time, and the first well in each row had 100 μL, or ~15 parasites. When the gradient was made, each successive well then should have had half the number of parasites as the well to its left. It was found that starting with 15 parasites in the first well was not a high enough number. The number was too small to make a consistent gradient, the wells ended up with widely varying numbers, and many wells received zero parasites. Therefore, the starting number of parasites per well was increased to ~25.

The controls tests involving the MTT assay were then performed as the final step before completing the anthelmintic tests. When doing the controls test, the MTT assay was found to not harm or kill the parasites. The parasites were left in the well for five days and there were still highly motile when examined under the microscope. However, when the isopropyl alcohol was added, the parasites did not turn the yellow solution blue, as they should have since they were visibly still alive and should have been metabolizing the MTT crystals. After discussing the problem, it was determined that the MTT assay was most likely not penetrating the cuticle.

The cuticle of the *Haemonchus contortus* parasite has three main outer layers made of collagen and other compounds, as can be seen in Figures B and C in the Appendix. The outer layers are non-cellular and are secreted by the epidermis. The cuticle layer protects the parasites so they can invade the digestive tracts of animals and not be affected by the acidic or toxic conditions found there. The L3 stage larvae also must survive on the blades of grass for hours to days on end, enduring the harsh rays of the sun or the cold night air. This indicates that the cuticle is built to endure many different environmental conditions, from sunlight, to cold, to acidity. In simple words, the cuticle is tough. The molecules of the MTT assay were not able to penetrate the tough layers of the cuticle in order to reach the cells and mitochondria of the parasite. Without mitochondria to process the MTT crystals, it appeared that the living, moving parasites were not metabolizing and were dead. After doing some research, it was discovered that there was another theory that would indicate why the MTT assay was not working with these particular parasites. The cuticle in the L3 stage larva surrounds their entire body, which covers their oral pore. The oral pore on this nematode is synonymous with the mouth on a human being. The oral pore is how the parasite ingests food, leading to the digestion of food, and ultimately the absorption and usage of nutrients. While in the L3 stage of the parasitic life cycle, the nematode cannot eat because the oral pore is covered. It was speculated that the lack of an operational oral pore might result in significant reduction in metabolic activity. The MTT assay is processed by the mitochondria in a parasite, but if there is little energy to be processed as there is no food in the body due to the sealing off of the oral pore, then the MTT assay might indicate the live, but idle, mitochondria as being dead. Either way, it was speculated that the cuticle may have been

the problem keeping the MTT test from working and keeping us from getting a positive control.

Information was found on a few different possible techniques for removing the cuticle. The first option involved washing the parasites in a diluted Clorox bleach solution and then centrifuging the discarded cuticles out of the solution. Determining the right concentration of bleach to use was a procedure that had to be added to this experiment, as there is not already a set procedure for removing the cuticle with bleach. Three original dilutions of bleach were made at 20x, 25x, and 30x dilutions of household bleach. Three centrifugation tubes were set up and each had equal parts of one of the diluted bleach solutions and parasites (~1mL). Each tube was left on a motion rotor, which kept the solutions in suspension while they were left to incubate at room temperature for 25 minutes. A drop from each tube was placed on to three different Petri dishes and watched under a dissection microscope to observe the shedding of the cuticles. A parasite sheds its cuticle, or goes through exsheathment, by creating a small tear by the head of the cuticle and using vigorous motion to remove itself. Visually, it looked like a snake shedding its skin (see Figure D, Appendix). The drops of solution containing the parasites were also observed to determine which dilution strength worked best, by removing the majority of the cuticles while not harming the parasites. Initially, the 30x dilution seemed too strong and the 20x solution seemed too weak, but all of the parasites in all three dilutions were plated in order to get more accurate final results. Before plating, each of the tubes went through the centrifugation process to remove as much bleach as possible from the solution. Each dilution had its own plate, and the 96-well plates were set up like the one shown below in Figure E and described in Table 2.

Figure E: The Setup of a 96-Well Plate When Testing the Controls

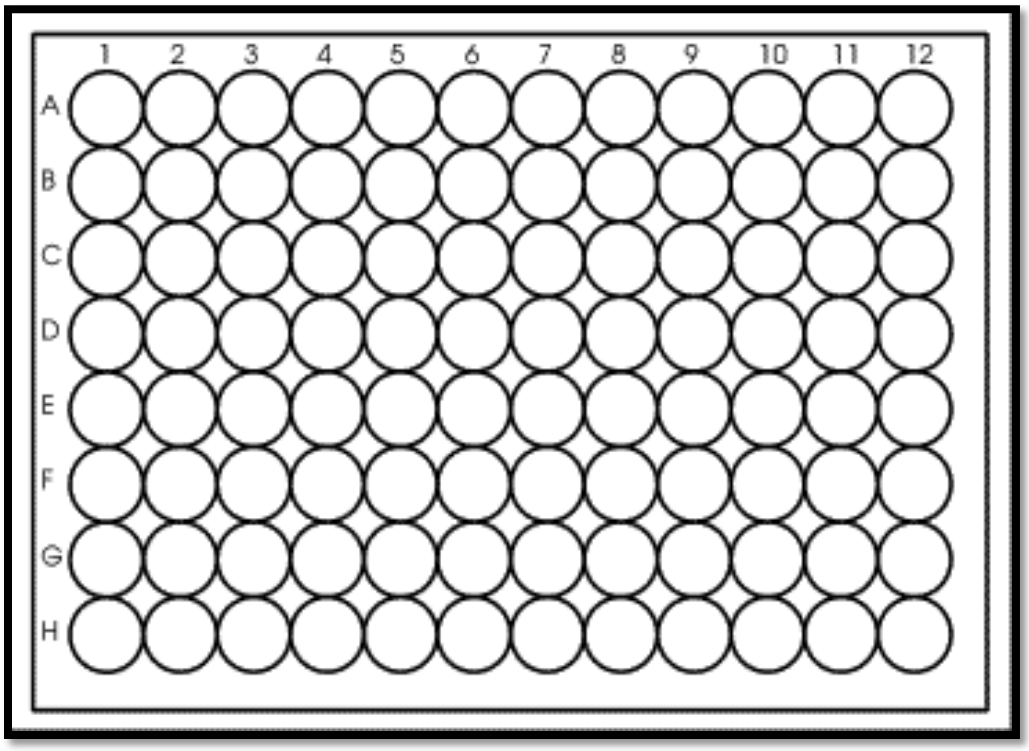


Table 2: The Setup of a 96-Well Plate When Testing the Controls

Row C	Parasites and MTT Assay
Row D	Parasites and MTT Assay
Row E	Only Parasites
Row F	Only Parasites

These control plates were incubated as described in the Materials and Methods section. When the plates were pulled out for analysis, the majority of the parasites in all of the wells were found to be dead.

It was deduced that there were two possible reasons that explained all of the parasites dying in the well plates over the course of three days. One reason may have been that the initial concentration of the bleach was too strong. They may have caused the

cuticle to be shed, but the parasites could not contend with the strength of the solutions and died. Another reason could have been that the centrifugation technique did not remove all traces of bleach from the solution before it was placed into the wells, and the residual (albeit low levels of) bleach caused the parasites to die over time. The specific cause of the deaths was not determined, but it was speculated that the second option was more likely to have occurred.

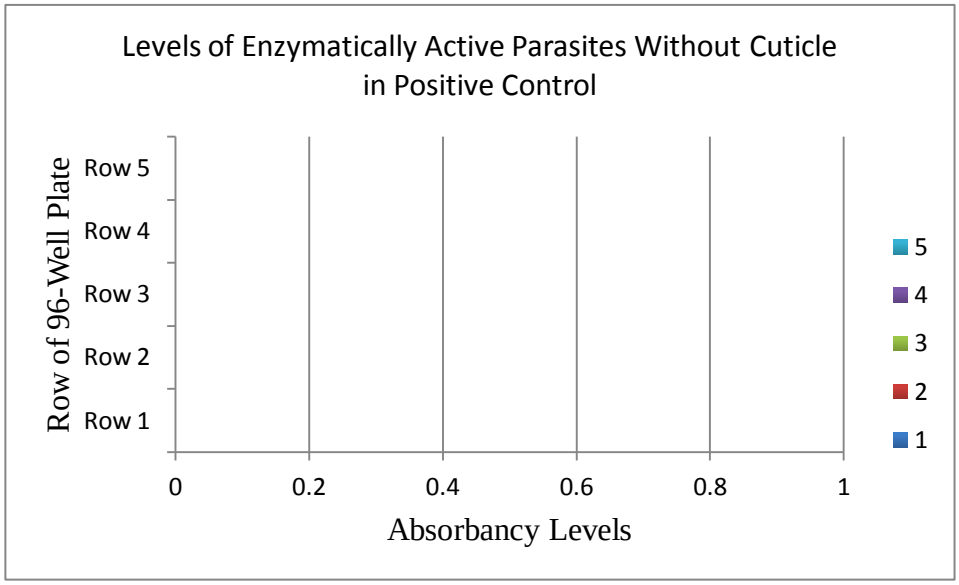
While doing research on solving the issues encountered with the bleach, another experiment was attempted with removing the cuticle. The conditions of the abomasum were attempted to be simulated in order to encourage the parasites to naturally shed their cuticles. In the natural life cycle of a *Haemonchus contortus*, the L3 stage larvae will be eaten by the sheep or goat and will then travel through the digestive system. Once in the digestive system, the cuticle is shed and the larvae progress to stage L4. In order to simulate the conditions found in the abomasum, the parasites were placed into a slightly acidic version of the PBS solution and then stored in the carbon dioxide incubator. These parasites were checked after one day, three days, five days, and nine days, but did not lose their cuticles. The parasites were all still alive and thrashing more than normal, but were still encapsulated by their cuticles. There may have been a factor that was overlooked and not included in the simulation, but the missing factor (if there is one) has not been discovered in the research.

When simulating the abomasal conditions did not work, the bleaching method was reattempted with a few modifications. In order to counteract the problems we originally encountered, sheets of nylon mesh with 31 micron openings were ordered. The pores in this nylon mesh sheeting are so small that the parasites should not be able to get

through it, but all liquid would pass through. It was also decided to use a much more diluted 2% bleach solution, which was made using 1 mL of bleach to 50 mL of water. This diluted bleach solution was incubated with the parasites. It was monitored under the dissection microscope for the shedding of cuticles, and once the parasites started shedding, one more minute was let pass before transferring the parasites to get rinsed. The shedding process took about 15 minutes total. The parasites were now rinsed using this new nylon sheeting in a set up similar to a Baermann Apparatus. The nylon sheeting replaced the Kimwipe in this instance, and the solution was poured out onto the nylon sheeting sitting in the top of the glass funnel. The parasites were all caught in the small pores of the mesh while all of the bleach and liquid ran out through the funnel. A beaker was then placed under the funnel, the nylon mesh was carefully flipped upside down so the parasites were on the bottom of the mesh facing the glass funnel, and PBS solution was used to rinse the parasites right into the beaker. Once this step was completed, the parasites were examined under a dissecting and a light microscope. Under the microscopes, it was determined that this method initially worked very well as all of the parasites had shed their cuticles and were still very motile. A little less than 20% of the parasites were unmoving and presumably dead, but that was the number observed to be dead before the bleaching. This high rate of dead parasites was most likely due to the fact that they had been living in culture in the refrigerator for almost two months. These parasites are very durable, but after two months a number of them would inevitably not survive for various reasons. These parasites without their cuticles were then tested in a 24-well plate in a similar manner to the 96-well setup seen in Figure E. There were two rows that included the MTT assay and two rows that housed only parasites. The plates

were incubated at 27 degrees C and were checked at day one, day three, and day six. On day six, it was found that the parasites were very motile, but there was no color change. As seen in Graph 1 below, the levels of color change were too low to even register and are therefore marked as zero across the board.

Graph 1: Levels of Enzymatically Active Parasites Without Cuticle in Positive Control



This graph clearly shows that, even after removing the cuticles of the parasites, the MTT assay did not work. It was not a good test to show the ratio of live to dead organisms within a well, as it did not show a color change when added to a well alone with parasites who were thrashing and moving as living parasites do.

It is important to mention one anomaly that occurred while testing the MTT assay portion of the experiment. During one attempt at getting a positive control, there were a few parasites that changed color from yellow to blue as were originally expected of the entire group, as seen in Figure F in the Appendix. It did not happen in all of the tests, or even in all of the wells of one plate, and the cause is not currently clear. This plate was one that was placed in the 37C carbon dioxide incubator, as it was temporarily available

at the time. It is not clear if the carbon dioxide incubator, or the increased temperature to 37C, caused the positive control to change color as it was supposed to or if it was another factor. Not all of the parasites in the plate changed color, which leads to the conclusion that it must have not been the carbon dioxide incubator or temperature as all of the parasites would have changed color. The only reason the incubator may have made the difference, however, would be if all of the plates would have changed color but something was wrong with the few wells that did not change. The carbon dioxide incubator was no longer available for use after this occurred, so this test was unable to be replicated, something recommended for future research in this project.

Unfortunately, the anthelmintic portion of this experiment, my hypothesis, and my second objective were not tested. It did not make sense to move forward and test the anthelmintic part of this experiment when a positive control could not be achieved. Without a positive control, there is nothing to compare the varying colorimetric levels of the wells to in order to determine the kill rate, or the live to dead ratio. Without a positive control, there is no way to determine if the MTT assay is working properly in the actual tests. It was decided to stop this experiment after the final failed positive control test, as it was necessary to rethink the strategy and the tests being used in this experiment. Despite not obtaining any results for the objective that was originally the main goal of this experiment, the second objective was explored and many useful techniques were discovered. It is a goal of the University of Maine Diagnostic Laboratory to be able to test the fecal samples sent to them by farmers around the state of Maine for parasites, especially the species and similar species to the *Haemonchus contortus* that were worked with in this experiment. The techniques that were researched and tested over the duration

of this project have either been incorporated or rejected for use by the diagnostic laboratory based in part on the results obtained from these experiments.

I would like to discuss some of my observations and thoughts for the future of this research, if it is continued in future years. Primarily, it is important to start early, preferably in the summer or very early fall. It will be much easier to obtain fecal samples that contain the parasite eggs if they are collected in a season when they are not dormant in the animals. The eggs of all abomasal parasites, including *Haemonchus contortus*, are laid in abundance in the late spring, summer, and early fall, as they are a subtropical species that thrive in the warm weather. The *Haemonchus contortus* parasites go into a hypobiosis during the winter months, and this is an important feature of their life cycle. Hypobiosis is a temporary halt in the parasitic phase of development at a specific point in the life cycle. Under normal circumstances when a host is infected with a nematode, parasitic development begins immediately and continues through to adult males and females in the normal period of time. However, under certain circumstances, larval development will be halted at a specific stage (usually L3 or L4 in this species) and the time spent in this stage is prolonged sometimes for weeks or months. Arrested larvae not only fail to grow but also their metabolic rate decreases significantly and they stop moving. In this state they can survive for weeks or months before resuming development, and this occurs in the winter months for this species. This hypobiosis state affects this research as no eggs are laid and shed in the feces when the parasites are in this arrested state. It would be very interesting, and helpful, if the samples were collected from many different farms in order to get a wide sample and more consistent numbers if presenting data on the state of Maine as a whole.

If future experiments are performed with a similar goal and methodology in mind, there are a few things that should be changed. If the cuticles of a group of parasites need to be removed, the method we used involving the nylon sheet filter worked very well. The only thing that should be added to the end of that procedure is that the newly washed and rinsed parasites, once the cuticles are gone and the bleach rinsed out, should be filtered through a Baermann setup in the same method used earlier. This would allow the motile L3 larvae to swim out through the Kimwipe and fall to the bottom of the glass funnel to be collected while the non-motile L3 larvae and cuticles remain in the Kimwipe, allowing for the solution and parasites to be kept much cleaner. During the initial rinsing, the cuticles do not fall through the nylon mesh, staying with the parasites and making it more difficult to differentiate the parasites from the cuticles. It would be easier and cleaner, as well as eliminate another unknown factor, if the entire solution was purified through another Baermann wash. The other thing that should change if trying to remove the cuticle is the size of the nylon mesh sheet ordered. A nylon sheet with pores the size of 20 microns would be preferable to the 31-micron sheet that was used. The 31-micron nylon sheet did work and kept most of the parasites from flowing through the filter, but many parasites, estimated at about 250, still were lost in the bleach solution. A 20-micron or smaller pore size would just keep the parasites from passing through the filter with less loss. The 20-micron nylon sheets were ordered once this was realized, but were on backorder. However, hopefully this means they will be in the lab available to whoever continues this research in the future.

The one change that will need to happen if this research is to continue is a switch to a different method of testing and determining the live to dead ratio. Without having a

way to determine the live-to-dead ratio, or the kill percentage, there is no way to test the effectiveness of the anthelmintics. It is obvious from this research project that the MTT assay does not work well enough for this experiment. If someone wanted to retest the MTT assay in the future, it would be beneficial to alter a major factor. In this project, the majority of the plates were incubated in the 27 degree C incubator. It might be worth testing to incubate the parasites in the 37 degree C carbon dioxide incubator to better simulate the conditions in which it normally lives in and metabolizes in without a cuticle. The carbon dioxide incubator was not available for the majority of my experiment, so it was not tested.

If pursuing new testing methods, there are many different ways that it could be done. It is important to find a method that is fairly inexpensive, easy to do and easy to replicate in different environments, not excessively complicated, yet accurate and effective. The MTT assay would have been a good choice if it had worked as it was expected to work. There have been a few research studies that have used fluorescent microspheres to great success when working with many organisms, but nematodes specifically. Fluorescent microspheres are microscopic (0.01μm) carboxylate-modified polystyrene beads that fluoresce to brilliant colors when observed under a fluorescent microscope (Ghafouri, 2006). It would be very interesting to use these beads in this experiment as they are relatively inexpensive, easy to use, and are proven to work with other species of nematode. The only expensive aspect of the microspheres is the need for a fluorescent microscope, which the University of Maine Hitchner laboratories are lucky to have. The beads could be used to test the live-to-dead ratio of the parasites after the drugs have been tested. Larvae would be incubated for a set amount of time in the

anthelmintic solution with ample food sources and fluorescent microspheres, and microspheres would likely be ingested along with similarly-sized E. coli. After incubation, the parasites would be placed on a slide and examined under a fluorescent microscope. The total number of nematodes seen on the slide, without fluorescence, could be counted; then the fluorescence could be turned on and the number of visible, fluorescent nematodes could be counted. The number of nematodes visible under fluorescence could be compared to the total number of nematodes visible without the fluorescence, which could determine the percentage of surviving nematodes. However, this assay will only work if the parasites are at a stage where they actively seek and eat food. Parasites at the L3 stage do not consume food as they are surrounded by the impenetrable cuticle and their oral pore is covered. These parasite larvae actively feed throughout the L1 and L2 stages, and might be better suited to this type of assay. The experiment performed by Ghafouri used these beads to great success and would be a good source if this technique is to be attempted in the future.

Another, more simple test could be used either as a supplement to the main assay or on its own. A chart could be derived by the person performing this experiment that compares the movement of the parasites to each other to result in a grade that represents the liveliness of the parasite. The chart is used under the assumption, and with the hope, that the movement of the parasites is directly related to the metabolic activity and the susceptibility of the parasite to the anthelmintic drugs, a higher grade indicating more liveliness and therefore more resistance to the drug being used. Originally, a chart was intended to be created with this idea in mind to supplement the MTT assay that was used in this research project. Unfortunately, this chart was never created as the MTT assay did

not work and there was no need for a supplemental chart test. A grading system created by me would not help whoever decides to continue with this project, as it would be entirely subjective based on my opinion and my observations. For example, what I consider to be a vigorously moving nematode might be seen as only a somewhat active nematode by another observer. The chart and grading system that was intended to be used for this particular experiment was designed to look similar to Table 3 below.

Table 3: Grading Scale Design for *Haemonchus contortus* Movement

Grade Scale	1	2	3	4	5
Type of Movement Observed	No movement	Slightly moving, but not very noticeable	Moving, but with many motionless breaks	Moving quickly with only short breaks	Vigorously thrashing or constant movement

It is easy to understand this grading scale, but it is very subjective, as only the creator of this chart truly understands what those different movements look like to their exact specifications, based on their own personal observations. The grading system chart would be beneficial as a supplemental tool, but would not be recommended as the sole test, as it is very subjective and time-consuming.

In conclusion, this experiment did not go exactly as planned. Many speed bumps came up throughout the process and the second objective, which originally started out as the focus of this experiment, could not be tested or completed. The hypothesis was not proved or disproved, and yet remains untested. This being said, many techniques were tested and either rejected or approved for future use, both in the future of this research and in the diagnostic laboratory. It was discovered that the MTT assay is not effective when working with these particular nematodes in this setting, although it is inconclusive to say whether that is because it is incapable of working in this experiment or if there

were certain factors that were overlooked. The methods for growing, purifying, and storing the parasites were perfected through many trials and can be used again when needed. The most important part of this experiment turned out to be the discovery that the removal of the L3 stage larval cuticle is possible, attainable, and fairly simple through the methods that were researched, altered to fit the needs of the project and laboratory, and used repeatedly. This project should be continued if at all possible, as many breakthroughs were made and it would be great to see it continued and more options explored, such as the ones laid out in the discussion.

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APPENDIX: FIGURES

Figure B: *Haemonchus contortus* Full Body with Cuticle

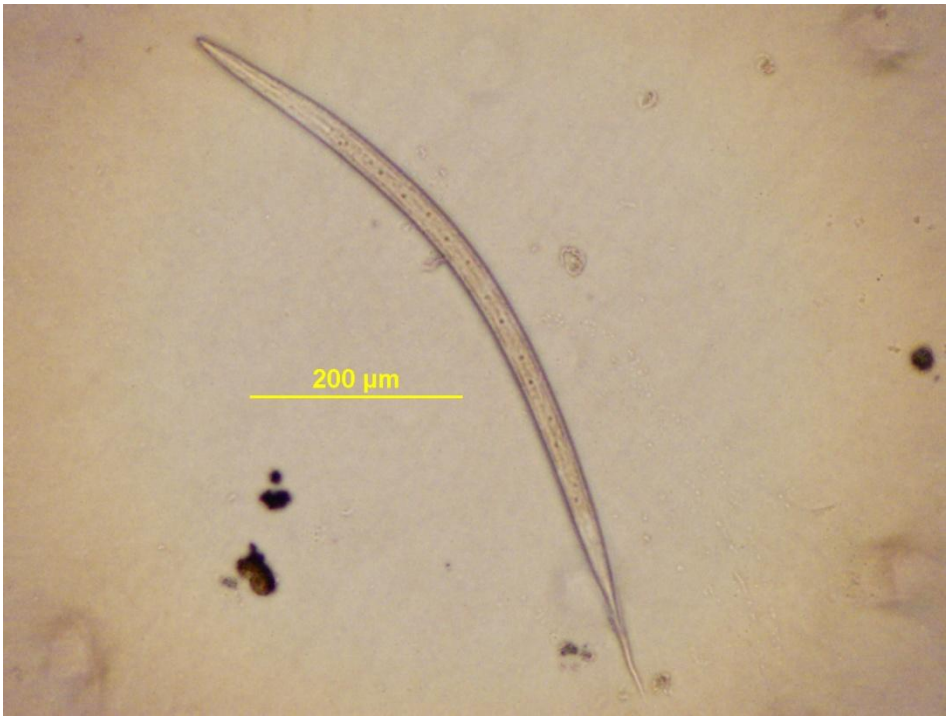


Figure C: *Haemonchus contortus* Tail, Illustrating the Cuticle

Figure D: *Haemonchus contortus* Cuticle after Shedding

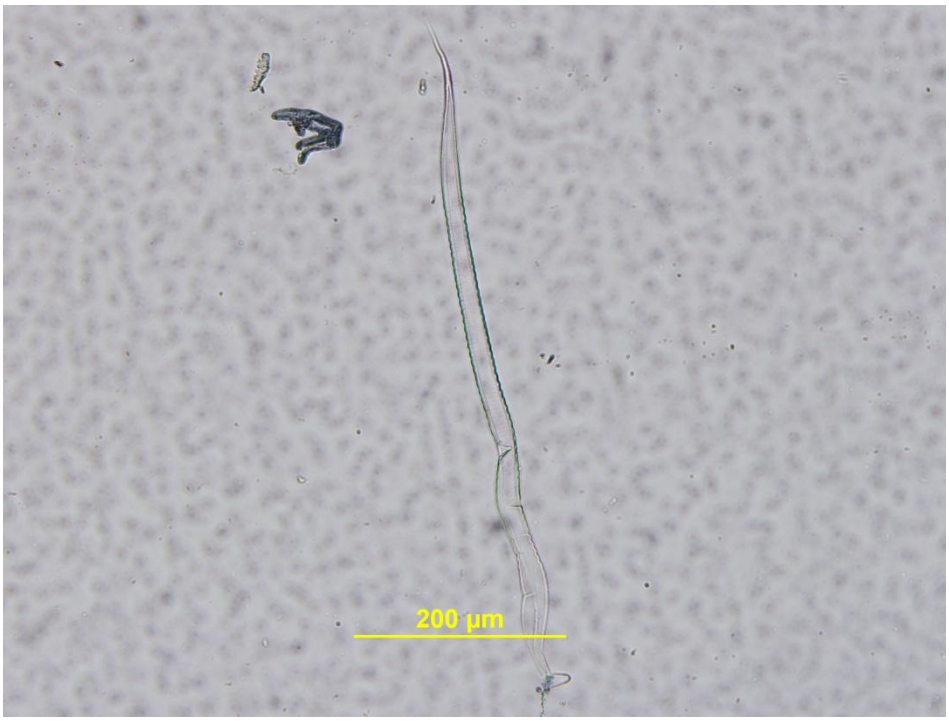
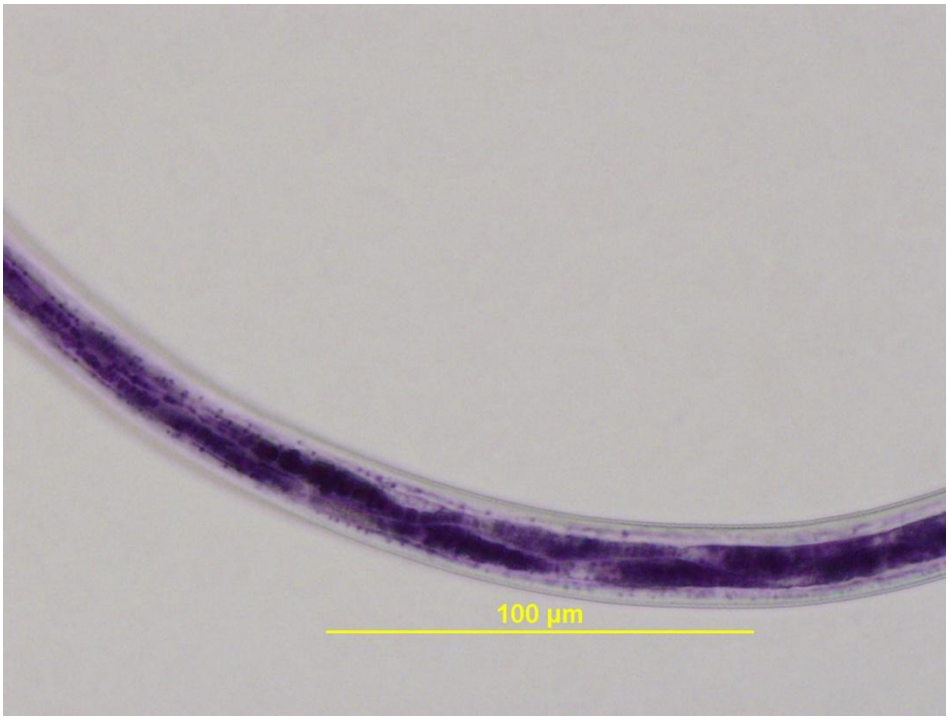


Figure F: *Haemonchus contortus*, Positive MTT Test



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Alexandra M. Settele was born in Coronado, California on July 1, 1990. She was raised in a military (Navy) family and grew up in many different areas of the world. She attended Naples American High School in Naples, Italy before moving and graduating from John Bapst Memorial High School in Bangor, Maine in 2008. Majoring in Animal Science, Alexandra has a concentration in Pre-Veterinary Medicine. She has been the President of Alternative Spring Break and the Vice President of All Maine Women.

Upon graduation, Alexandra plans to attend veterinary school at the Ross School of Veterinary Medicine or graduate school at the University of New Hampshire for a Masters Degree in Microbiology, once she makes a decision. She would eventually like to become a practicing large animal veterinarian.