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drugs on the growth and development of the camelid parasite,
Eimeria macusaniensis, in cell cultures**

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Techniques for determining efficacy of different anti-coccidia drugs on the growth and development of the camelid parasite, *Eimeria macusaniensis*, in cell cultures

Daniel Houlihan

Abstract

Eimeria macusaniensis, a coccidian parasite, continues to be a threat to New World Camelids (NWCs) in the United States because there are no current proven methods of treatment. This internal parasite is a major cause of diarrhea in NWCs, invading the small intestine of NWCs and increasing the susceptibility for secondary bacterial and viral infections. Clinical signs of infected animals range from mild diarrhea and decreased appetite to death. Currently used treatment protocols for *E. macusaniensis* coccidiosis in llamas and alpacas have been extracted from those used to treat non-camelid species against coccidian, as data and validated treatment protocols in NWCs are lacking. This research project studied the life cycle of *E. macusaniensis* in a laboratory setting by examining and modifying various techniques used to detect, concentrate, and sporulate *E. macusaniensis* oocysts for use in future research projects. The new protocols and modifications developed in this project will lay the foundation for a later project in which infectious sporozoites obtained from sporulated *E. macusaniensis* will be inoculated in vitro in Vero-cell cultures. Once inoculated, the infected cultures will be subjected to a drug dosing assay of various anti-protozoal drugs to test the efficacy of the drugs on the destruction of the parasite. The completion of this project provides enough evidence to support further in vivo/vetro testing in the treatment of *E. macusaniensis* in llamas and alpacas.

Eimeria macusaniensis is a protozoal parasite of the Phylum Apicomplexa that causes fatal enteritis in New World camelids (NWCs) worldwide (Rickard 1994; Lenghaus, O'Callaghan et al. 2004; Cebra, Valentine et al. 2007). Infected animals will shed oocysts in manure, which eventually sporulate in the proper environmental conditions. These infective sporozoites are then taken up by other foraging NWCs and continue the life cycle in the small intestine of the host. Infectious sporozoites invade host cells and form merozoites through an internal fission process called schizogony. Oocysts are released by rupture of the host

cell and pass into the feces (Bowman). Because the majority of intestinal damage occurs prior to shedding oocysts, disease and clinical signs can be apparent before oocysts are even detected in feces. In most instances, a healthy adult animal with a mild infection of *E. macusaniensis* will rarely demonstrate clinical signs of infection. However, in immuno-compromised, young, old or severely infected animals, infections are associated with lethargy, anorexia, weight loss, sudden death and hemorrhagic diarrhea (Ballweber 2009).

E. macusaniensis may measure up to twice the size of other NWC specific

Eimeria, measuring 93.6 μm by 67.4 μm compared to a range of 30-60 μm by 30-40 μm of other *Eimeria*. This large size variance can lead to severe clinic signs even in healthy animals (Cafrune et al. 2009). Unlike most coccidian in which infection is limited to one hosts, *E.macusaniensis* has been shown to effect all four NWC species (llamas, alpacas, vicunas and guanacos) (Leguia 1991;Beldomenico, Uhart et al. 2003). Due to its large size, it is more likely to result in devastating secondary bacterial and viral infections in the small intestine. These secondary infections cause the animal to experience moderate to severe diarrhea and can often lead to death after exhibiting clinical signs for only a few days. Since *E.macusaniensis* could pose a large threat to farmers in the United States (especially in the Midwest), a successful and effective form of treatment is vital. This paper will explore various protocols for the steps involved in producing viable and infectious *E.macusaniensis* for use in Vero-cell cultures.

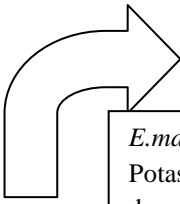


Figure 1: *E.macusaniensis* compared to a typical gastro-intestinal nematode egg (GIN)

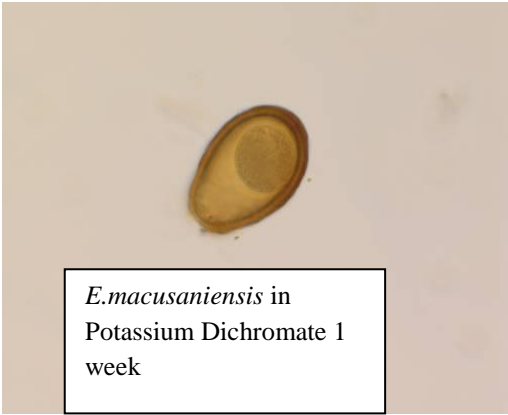
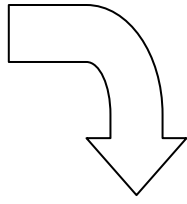
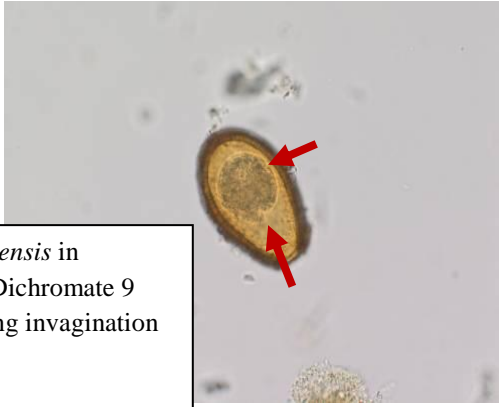
Materials and Methods

In order to produce infectious *E.macusaniensis* sporozoites, a constant supply of viable oocysts needed to be obtained. Initially, samples of fresh llama and alpaca feces were evaluated for the presence of *E.macusaniensis* from various farms in Virginia and Tennessee (Kinser and Dreamland Farms) over the years 2008-2009. Samples were collected by herd owners, shipped chilled and examined within one week of arrival at the University of Tennessee College of Veterinary Medicine Parasitology Diagnostics Laboratory. Fecal samples were stored in zip-lock bags at 3-4°C when not being used. In order to test

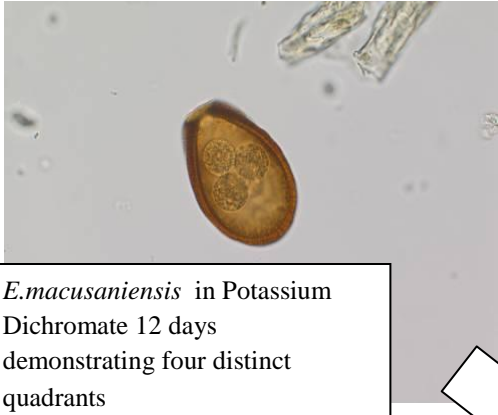
for the presence of *E.macusaniensis*, 1-2 grams of a sample were mixed with 15 ml of distilled water. The solution was filtered through 2-layers of grade #50 cheesecloth (approximate filtration of 100 μm), placed into a 15 ml centrifuge tube, and centrifuged at 1500 rpm for 5 minutes. The supernatant was decanted and discarded without disturbing the pellet. The pellet was then reconstituted and mixed with Sheather's Sugar Solution (spg. 1.275) and filled to the top (slight meniscus present). A cover slip was placed onto the centrifuge tube and it was centrifuged at the above conditions. Upon completion, the sample was examined



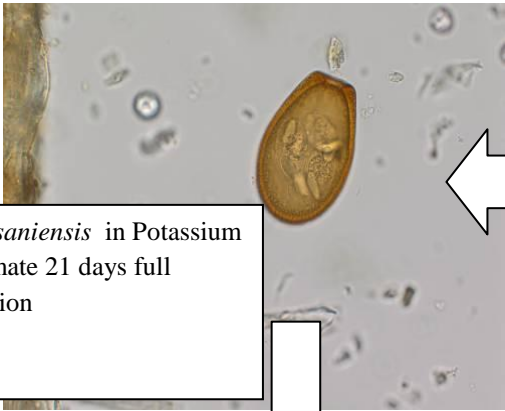
E.macusaniensis in Potassium Dichromate 9 days showing invagination



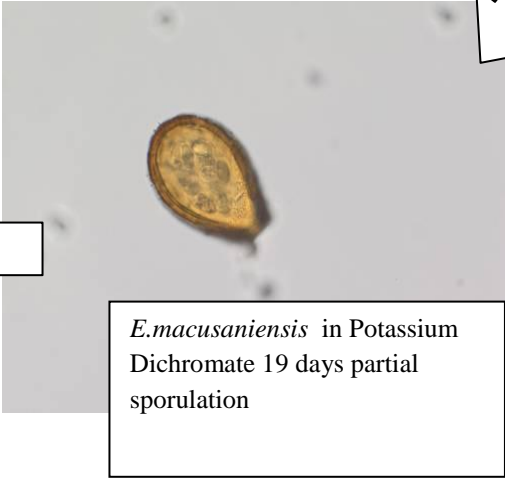
E.macusaniensis in Potassium Dichromate 1 week



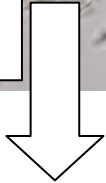
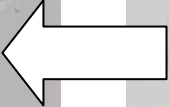
E.macusaniensis in Potassium Dichromate 12 days demonstrating four distinct quadrants



E.macusaniensis in Potassium Dichromate 21 days full sporulation



E.macusaniensis in Potassium Dichromate 19 days partial sporulation



Infection if ingested

under a microscope at 100x power to check for the presence of *E.macusaniensis*.

Unfortunately, a lack of positive samples (approximately 2 cases out of 100) from farms on the East Coast temporarily impeded research efforts. To ensure a constant supply of *E.macusaniensis*, two llamas were obtained from a local stock sale for use in this project. The llamas, (estimated age approximately 3 years) were housed at the East Tennessee Clinical Research Center in individual stalls under constant veterinary care. Upon examination using the methods listed above, it was discovered that one of the llamas had a pre-existing *E.macusaniensis* infection. Samples from this llama were collected every 1-3 days and stored at 3-4°C until a float could be done to confirm the presence of *E.macusaniensis*. Oocyst/gram counts from positive samples were estimated using the Wisconsin Double-Coverslip method.

Once a positive sample with sufficient oocysts/gram (15-35+ oocysts/g of fecal sample) was found, the sample had to be concentrated down. Multiple protocols, including various forms of filtration and solutions (concentrated saline (spg 1.195) and Sheather's Sugar without preservative (spg 1.275)) were explored to find the most efficient method of *E.macusaniensis* oocysts concentration. After numerous combinations were explored, the following method proved to be most useful. Samples (stored in a gallon sized zip lock bag) were mixed with approximately 1-1.5 liters of warm tap water. The feces were then crushed and mixed with the water and let to sit for 20 minutes. The homogenous solution was then filtered and strained through 3 layers of grade #50 cheesecloth (discard remaining solid). The solution was then filtered through a standard sieve (#400 38 µm). The solid left behind in the sieve was washed with distilled water into multiple 50 ml centrifuge tubes. The

centrifuge tubes were spun at 1500 rpm for 8 minutes and the supernatant was decanted without disturbing the pellet. The tubes were then filled back with tap water and re-spun at the above conditions as a wash until the supernatant was mostly clear. Sheather's sugar solution (spg 1.275)



Figure 2: *E.macusaniensis* demonstrating four distinct sporoblasts after 12 days in Potassium Dichromate

without preservative (Formaldehyde) was then poured into the tube and mixed with the pellet. Each tube was then centrifuged again at the above conditions. The top 3-5 mls of each tube was suctioned off and poured into a single 50 ml centrifuge tube, making sure to not have a single 50 ml tube with more than 20 mls of supernatant (50 ml tubes with sugar were saved for a later time). The tube was then filled with tap water and centrifuged a final time (1500 rpm for 5 minutes); pouring off the supernatant (the pellet should now be highly concentrated with *E.macusaniensis* oocysts). The tubes in which the top layer was siphoned off were then stored at 3-4°C for 3-5 days allowing time for all *E.macusaniensis* oocysts to float to the top. The process was then repeated starting with the siphoning of the top 3-5 mls of solution. Concentrated E.mac was then stored in 4% potassium dichromate in 3-4°C until used in sporulation.

Sporulation of *E.macusaniensis* was also attempted with various protocols to find the most efficient method. Most Eimeria typically sporulate at room temperature in 5-7 days when mixed with 2.5-4% potassium dichromate. However, from preliminary data, it was found that *E.macusaniensis* takes approximately 2-4 weeks to reach its infectious form in the laboratory at the above conditions. In an attempt to make the process more efficient, various factors were changed to decrease sporulation time. Both 2% sulfuric acid and 4% potassium dichromate were compared in the sporulation process. Similarly, samples were tested at both room temperature and at approximately 27.3 °C (81°F) in an incubator. Samples were placed into a beaker with 400 ml of 4% potassium dichromate (with an escape valve) and aerated with a constant supply of air from a fish tank aerator and tubing. During the sporulation process, water was added to account for lost water during evaporation. The sample was checked every few days to monitor the sporulation process. Completely sporulated and infectious *E.macusaniensis* will be used to re-infect the llamas to provide a constant supply of oocysts or will be stored for later use in vero-cell inoculation.

Results

After multiple attempts at perfecting the protocols for concentrating and sporulating *E.macusaniensis*, a few procedures appeared more successful than others. The initial concentration method attempted with a concentrated saline solution (spg 1.195) proved to be successful (Hofmann et al. 1990). However, the process prevented concentration of large quantities of feces (limited to 200 grams) and was inefficient as a mass concentration method. The method described above, using a non-preservative form of Sheather's sugar

(spg 1.275) demonstrated similar success with the ability to concentrate large quantities of feces (upwards of 700 grams) at once. Similarly, the amount of centrifugation for the second method was lower. This particular method resulted in a collected concentration of ~45% (roughly 4,000 oocysts out of an estimated 8,900.) of the oocysts as determined by the Wisconsin Double cover-slip method after the first siphoning. When the 50 ml tubes were allowed to sit for 3-5 days, the total collection numbers jumped to 90% of total estimated oocysts. In order to increase time and efficiency of this procedure, it is recommended that the #400 standard sieve be replaced by a #270 (53 µm) in order to still ensure collection of *E.macusaniensis* (measuring 93.6 µm by 67.4 µm) while decreasing time length of filtration.

Similar to the concentration protocols, two protocols for sporulation were tested. The first used a 2% sulfuric acid incubated at a temperature of 27.3 °C (81°F). Multiple cell culture flasks (25cm³) with positive fecal samples were placed onto an orbital shaker at 380 rpm. Caps were left loose in order to allow for an exchange of oxygen. After 8 days, oocysts started showing signs of sporulation. Complete sporulation was observed by day 28.

The method, described in the material and methods section of this paper, which used a 4% potassium dichromate solution instead of 2% hydrochloric acid demonstrated slightly faster results, showing compartmentalization by day 7 and complete sporulation by day 21 of the process. Similarly, it was found that when incubated, the solution of potassium dichromate created less fumes than that of the hydrochloric acid. Due to the faster sporulation time and decreased fumes, it is recommended that the protocol with potassium dichromate incubated at 27.3 °C (81°F) be used.

Discussion

Clearly, there is a need to continue research on this particular NWC parasite. With little information available on this specific coccidian, it becomes difficult to diagnose and treat. This paper, which reviewed and modified protocols established for other species of *Eimeria*, will be used to lay the foundation for a larger research project in which sporulated *E. macusaniensis* will be inoculated into cultured vero-cells to test the efficacy of various anti-coccidian drugs. By following the protocols laid out in the materials and methods section of this paper, one will be able to locate, concentrate

and sporulate *E. macusaniensis* in an efficient and timely manner for use in further research and study of this coccidia.

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