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An Assessment Of Hookworm Infection And Albendazole Treatment Failure Among Children Ages 7-12 In Kintampo North Municipality, Ghana

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Abstract

Background

Hookworm is a soil-transmitted nematode (STN) infection associated with rural poverty that infects 576-740 million people worldwide, 200 million of which reside in Sub-Saharan Africa. In 2001, the WHO passed a resolution that recommended annual deworming for school-aged children where the prevalence of STNs is higher than 20%. Since this time, albendazole has been widely used across sub-Saharan Africa to treat hookworm infection. Given the widespread use of albendazole as a treatment for hookworms and other STNs in humans, it becomes pertinent to ensure that resistance is monitored at the local level, and detected as it emerges. Previous research in Kintampo, Ghana found a high prevalence of low intensity hookworm infection prior to treatment, and raised concerns about emerging resistance to albendazole.

Methods

Children between the ages of 7-12 from four villages were enrolled in this study and given a questionnaire to gather demographic and health information about each participant (n=178). Fecal containers were distributed to enrolled children and the Kato-Katz technique was used to identify a positive infection and to estimate the severity of the infection. Children that were infected with hookworm were treated with a single dose of 400mg albendazole, and a second stool sample was collected from these children at 10-14 days post-treatment. Kato-Katz methods, Egg Extraction, EHA, and the Baermann method were conducted on all positive samples pre- and post-treatment. Genomic DNA was extracted from frozen purified hookworm eggs and larvae from pre- and post-treatment samples and stored for future testing. Molecular methods were utilized to identify the hookworm species in each sample

Results

At baseline, 57/178 (32.0%) of children from four villages were positive for hookworm. The highest prevalence was seen in Jato (43.7%), while the lowest prevalence was observed in Tahiru (8.0%). Post-treatment, 36.8% (21/57) of children infected at baseline were still hookworm positive. All of the children from Cheranda and Tahiru were cleared of infection, while in Mahama, one child harbored a light infection (CR=80%, FECR=97.9%). In Jato the cure rate was low (55.5%) and the Fecal Egg Count Reduction rate (FECR) was suboptimal (87.9%). Pre-treatment, almost all children harbored light infections, while all children were lightly infected post-treatment.

Discussion

These data demonstrate the need for more targeted approaches to the treatment of helminth infections, as variable responses are observed within each community. Findings from Jato suggest that in some communities, therapeutic intervention alone is not enough. Control measures such as health education or providing access to latrines could make a more substantial impact in communities like Jato, where MDA has been implemented, and where infection rates continue to be high among school-aged children. Data from this study further support the need for new approaches to combat the disease burden posed by helminth infections in much of the developing world.

Acknowledgments

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I. Introduction

a. A Brief History of Hookworm Disease

Today a disease of rural poverty, hookworm infection has been observed in humans for over 5,000 years (Cox 2002). The earliest written record of hookworm infection is commonly ascribed to the Ebers papyrus of 1500 B.C., when an anemia like condition that caused pallor and laziness was described (Cox 2002). Records from 300 B.C. depict hookworm infection as a disease that is characterized by intestinal distress and a tendency to eat dirt (Power 2001). Similar descriptions of a disease associated with pallor and weakness appear in the writing of Lucretius in 50 B.C., and in references from China in the third century(Cox 2002). An increasing number of references to the suspected disease appeared in records from European explorers in the West Indies and South and Central America in the 18th-19th centuries (Cox 2002).

Italian physician Angelo Dubini first identified one species of hookworm, *Ancylostoma duodenale*, in 1838, where he observed that it used its teeth to attach to the intestinal lining (Power 2001). Building off his work, Wilhelm Griesinger drew a connection between the worms and iron deficiency anemia during autopsies in 1854 (Cox 2002). While examining feces with microscopy in 1878, Giovanni B. Grassi developed the first diagnostic method for examining hookworm ova, and the first anthelminthic drug, thymol, was developed shortly thereafter (Power 2001). Just two years later, Edoardo Perroncito correlated an anemia outbreak among miners in the St. Gothard tunnel with hookworm infection, after noting in autopsies that a high number of hookworms correlated with the symptoms of anemia (Power 2001). Finally, in the early 20th century, a second species of hookworm, *Necator americanus* was identified among agricultural laborers in Puerto Rico. Shortly thereafter, Arthur Loos

discovered how hookworms enter the body and reach the small intestine after accidently infecting himself (Power 2001, Cox 2002).

b. Hookworm Biology and Disease Pathology

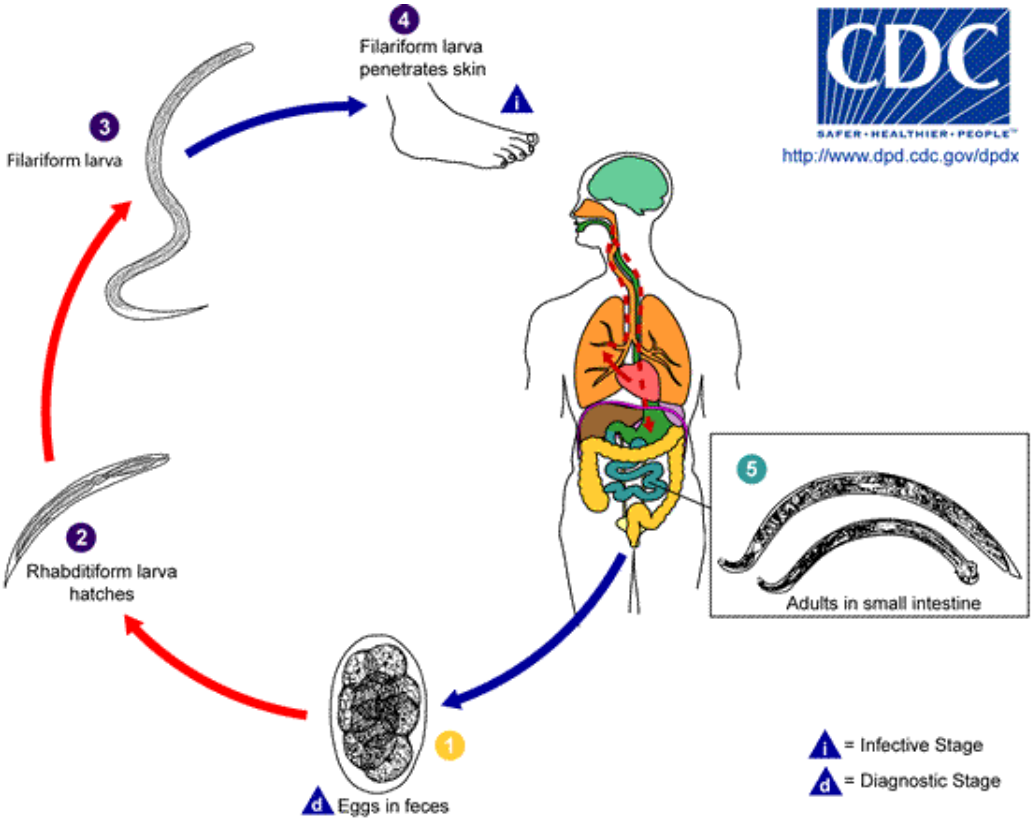
Human hookworm infection is a parasitic disease caused by two types of blood sucking, soil-transmitted nematodes (STNs): *Ancylostoma duodenale* and *Necator americanus* (Hotez P. 2005). Symptoms of infection vary, and typically begin with itching and a localized rash that results when larvae penetrate the skin. Although most infections with hookworm are asymptomatic (especially light infections), symptoms may include abdominal discomfort, fatigue, weight loss, anemia and protein deficiencies. Heavy infection can result in the impairment of physical and cognitive development, especially among children, who can lose up to 20mls of blood each day as a result of infection (Humphries D 2012, Bird, Ame et al. 2014).

Although infection with hookworm is rarely fatal, it can often increase susceptibility to other STNs such as trichuriasis, and diseases such as malaria, HIV and tuberculosis (Humphries D 2013). The impact of hookworm infection on the host, and the level of anemia that results are typically dependent on a variety of factors. The worm burden plays a role in influencing the severity of disease, as does the hosts' nutritional status. Iron reserves and diet play a key role in the severity of disease, making the infection more detrimental among children that are malnourished. The species of hookworm also plays a role in the severity of disease, due to the fact that *A. duodenale* typically causes more blood loss than *N. americanus* (Brooker S 2004).

c. Hookworm Lifecycle

When hookworm eggs are passed in the stool, under certain conditions (such as shade, moisture and warmth) the eggs will hatch over a period of 1 to 2 days ((CDC) 2013). Over a period of 5-10 days, the hatched larvae undergo two molts, after which time they become infectious filariform third-stage larvae (L3) ((CDC) 2013).Under the right environmental conditions, these larvae can live in the soil for as long as 4 weeks ((CDC) 2013).

Figure 1: Life Cycle of Human Hookworms



Humans typically become infected with hookworm when skin comes into contact with L3 larvae in the soil, although *A. duodenale* L3 have been shown to infect by both the skin and oral route (Hotez P 2004). Following host entry, L3 are carried by blood vessels to the heart and then on to the lungs over a period of approximately ten days ((CDC) 2013). L3 enter the digestive system by penetrating the pulmonary alveoli and ascending to the trachea, where they are coughed up and swallowed (Hotez P. 2005, Bungiro R 2011). Upon entering the

gastrointestinal tract, hookworms undergo two molts, after which time they reach the adult blood feeding stage (Hotez P 2004). After using their teeth to attach to the intestinal mucosa in the lumen of the small intestine, they begin feeding on tissue and blood (Hotez P. 2005, Bungiro R 2011). Once adhered to the small intestine, adult hookworms mate and begin producing eggs, which are then excreted in the feces (Hotez P. 2005, Bungiro R 2011). Following excretion, the hookworm eggs in the stool are released into the environment, and can thereby repeat the parent life cycle (Hotez P. 2005, Bungiro R 2011) (Figure 1).

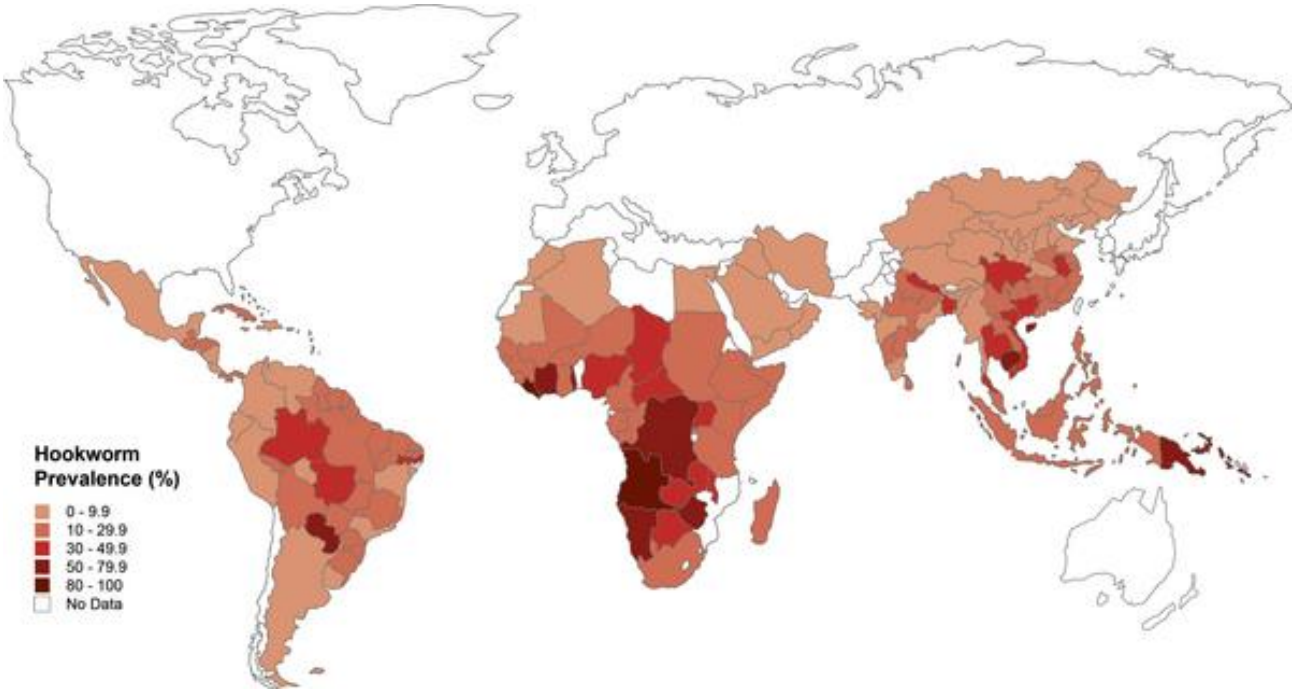
d. Epidemiology of Hookworm Infection

Although hookworm infection has been eliminated from more developed nations, infection is still highly prevalent in many parts of the developing world, especially among school-aged children (SAC). It has been estimated that there are still an estimated 740 million people infected with hookworm around the world, with more disability adjusted life years (DALYs) lost than any other helminth infection except lymphatic filariasis (Hotez P 2006). Higher prevalences of disease are observed in tropics and subtropics, and highest in rural areas (Brooker S 2004). Although it is more common to see the highest intensities of STN infections among children, both children and adults are frequently heavily infected with hookworm (Hotez P. 2005).

Infection with *N. americanus* has been shown to be more widespread, while infection with *A. duodenale* has been shown to be more focalized (Hotez P. 2005). Infection with *N. americanus* is seen throughout the world, in regions such as South and Southwest China, Southeast asia, Southern India, sub-Saharan Africa, and South and Central America (Brooker S 2004). In contrast, *A. duodenale* is predominantly found in regions with harsher environmental conditions, where it is less likely for *N. americanus* to survive (Brooker S 2004). These regions include the northern parts of southern India, southern and western

China, and a few parts of Latin America (Northern Argentina, Paraguay) and Northern Australia (Hotez P. 2005).

Figure 2: Global Distribution of Human Hookworm Infection



Hotez PJ, Bethony J, Bottazzi ME, Brooker S, et al. (2005) Hookworm: "The Great Infection of Mankind". PLoS Med 2(3): e67.

While hookworm infection can be found in much of the developing world, rates vary by region (Figure 2). Central and South America see the lowest prevalence of disease (10%), followed by China and south Asia (16%) and southeast Asia (26%) (Humphries D 2012). The highest prevalence and intensity of infections are observed in Sub-Saharan Africa (29%), where there are an estimated 200 million cases (Brooker S 2004, Humphries D 2011). While improvements in socioeconomic status, water, sanitation, and control efforts have decreased the disease burden in other parts of the world such as Latin America, there has been little change in the prevalence of hookworm in Sub-Saharan Africa (Brooker S 2004). In Ghana, some regions see a prevalence of infection that exceeds 87.5%, while rates of infection

among school aged children in Kintampo have been reported to be around 45% (Ziem J 2006, Humphries D 2011).

e. Risk Factors for Infection

Hookworm infection transmission is dependent upon environmental contamination with hookworm eggs. As such, infection with hookworm is often influenced by socioeconomic status. Limited access to clean water, sanitation and adequate health care, along with poor education provide an environment under which transmission can continue (Hotez P 2004). Working in agriculture, a common occupation in much of the developing world, has been shown to be associated with infection, especially in areas where waste water or night soil is regularly used to enhance agricultural production (de Silva 2003). Inadequate hygiene practices and the absence of latrines and sewage treatment have also been shown to be associated with infection (Raso, Vounatsou et al. 2006). Not wearing shoes is also associated with elevated rates of infection, and poor nutritional status, malaria, and anemia prior to infection are known to exacerbate the effects of the disease (Hotez P 2004).

f. Disease Treatment and Management: Efficacy of Benzimidazoles and Secondary Drugs

The main class of drugs used to treat soil-transmitted helminthes, including hookworm infections, is the benzimidazoles. These drugs bind to the tubulin protein of microtubules, which are important organelles involved in cell motility and division (Keiser J 2010). By binding to these organelles, the uptake of glucose is blocked, which ultimately empties glycogen reserves in the parasite (J 2002). Depleted of energy, the worm is then expelled from its host, or is paralyzed and dies (J 2002). Although lethal to the parasite, these drugs are generally well tolerated by the host (J 2002). This is because they have a higher affinity for binding to tubulin in the worm than in humans or livestock (J 2002). Although

considerably effective at treating many types of worm infections, these drugs are quickly excreted from the body and do not provide any protection against reinfection (J 2000).

Albendazole, a drug in the class of benzimidazoles, is the primary drug recommended for the treatment of hookworm infection. Approved for human use in 1987, albendazole is administered orally and can be used to treat a variety of worm infections, including ascariasis, trichuriasis, enterobiasis and hookworm (Keiser J 2010). In a recent review conducted by Keiser and Utzinger, 11 different studies showed that albendazole therapy was well tolerated, and that there were no significant adverse events reported following albendazole administration (Keiser J 2008). However, mild symptoms including dizziness, headache, nausea, vomiting, and abdominal pain have been reported in response to treatment with a single dose of albendazole (Keiser J 2010).

Mebendazole is another benzimidazole that can be used to treat hookworm infection, although the drug is not as effective at treating hookworm as albendazole (Keiser J 2010). Like many other benzimidazoles, mebendazole is administered in a single dose and targets the tubulin in the parasite cell (Keiser and Utzinger 2008). Although mebendazole is less effective in the treatment of hookworm infection, it has a high efficacy in the treatment of Ascaris (Keiser and Utzinger 2008).

Pyrantel pamoate acts as a nicotinic acetylcholine receptor antagonist in the parasite (Keiser J 2010). By doing so, it causes spastic paralysis of the worm, ultimately leading to death.(Keiser J 2010) Although it is a second line drug for hookworm, pyrantel pamoate is more commonly used in the treatment of ascariasis and enterobiasis, with cure rates of 90-100% (Keiser and Utzinger 2008). In a review examining its efficacy in treating hookworm infection, nearly half of patients in one trial experienced adverse events such as nausea, dizziness and abdominal pain (Keiser and Utzinger 2008). Similar to pyrantel pamoate, levamisole targets the acetylcholine receptor within the parasite, ultimately leading to the

paralysis and death of the worm. Levamisole is associated with a diverse array of side effects including vomiting, diarrhea, dizziness, and headache (Mehlorn 2008). Given the higher risk of adverse events, these drugs are recommended only as second line drugs for the treatment of hookworm infection (Keiser and Utzinger 2008).

g. An Assessment of Drug Efficacy and the Treatment of Hookworm Infection

When examining the efficacy of these drugs against hookworm in 20 randomized controlled trials, a single dose of 400 mg albendazole was found to have an overall cure rate of 72%, while mebendazole had a cure rate of 15% and pyrantel had a cure rate of 32% (Keiser J 2008). Levamisole treatment in several trials had cure rates between 10%-38% (Keiser J 2008). This study went on to conclude that, when administered as a single-dose therapy, albendazole reduced the prevalence of hookworm more effectively than any other drug (Keiser J 2008). Although albendazole is the primary therapy used to treat hookworm infection, pyrantel pamoate and levamisole are considered to be alternative treatments for hookworm (Keiser J 2008). Despite the low cure rate for mebendazole therapy, it is still widely used to treat hookworm (Keiser J 2008). In Ghana, between 4-5 million children are still treated with mebendazole therapy each year, despite its low cure rate (Keiser J 2008).

There have been several studies that have examined the decreasing susceptibility of parasitic worms to anthelmintic drugs in livestock. A recent study conducted in Uganda observed very low efficacy for albendazole therapy (28.5%) when treating goats for gastrointestinal nemotodes (Byaruhanga C 2013). Another study conducted in Northern Ireland examined treatment efficacy for several benzimidazoles used to treat flocks of sheep, and found significant resistance to one or more of these drugs in 81% of blocks tested (McMahon C 2013). In a 2010 study, Vercruysse points out that many years of using anthelminthic drugs to control roundworms in livestock resulted in high levels of resistance

to the drugs (Vercruysse J 2011). Later, he mentions that treatment frequency and possible under-dosing have been identified as contributors to the development of drug resistance (Vercruysse J 2011).

Although albendazole therapy has become the standard course of treatment for hookworm infection in humans, there have been reports that have brought up the possibility of a decreasing susceptibility to the drug. A study conducted in Laos in 2010 noted that albendazole therapy had a cure rate of 36%, far less than the cure rate seen in the majority of studies (Soukhathammavong P 2012). While they concluded that differences in hookworm species susceptibilities, host factors, and co-infection with other STNs could play a role in treatment failure, the possibility of increasing resistance to albendazole could not be ruled out (Soukhathammavong P 2012). Two trials conducted in Vietnam in 2007 found that single-dose albendazole was no more effective against hookworm than a placebo, and noted that only a three-dose regimen of albendazole was more effective at treating hookworm than the placebo (Flohr C 2007).

h. Strategies for the Control of Disease Burden

At the turn of the 20th century, hookworm disease, commonly referred to as a "disease of laziness" was widespread across the southern United States (Bleakley 2007). After realizing the serious public health problem that hookworm disease posed in this region, the Rockefeller Foundation founded the Rockefeller Sanitary Commission (RSC) for the eradication of hookworm disease, and initiated a campaign from 1910-1915 (Brooker S 2004, Bleakley 2007). This campaign used a multifaceted approach to decrease the burden of the disease, involving treatment, education, and latrine building across the southern United States (Bleakley 2007). At the start of the campaign, the RSC surveyed over 600 counties across the south and found that the prevalence of disease among school aged children in the American

South averaged 43% (Bleakley 2007, M 2009). Following this observation, the RSC built latrines and created traveling treatment dispensaries that provided thymol to nearly 400,000 individuals over the course of the campaign (Power 2001, Bleakley 2007, M 2009). At the same time, the RSC initiated an educational campaign to inform both the public and local physicians about hygiene practices and how to recognize and prevent infection (Bleakley 2007). Although these strategies failed to eliminate hookworm disease from the southern United States, this multifaceted approach resulted in a 50% decrease in the prevalence of the disease (Bleakley 2007). As the RSC's campaign came to a close, local and state governments began to fund anti-hookworm campaigns, and took over many of the foundations activities (Bleakley 2007, M 2009). As result of this campaign and the growing awareness that ensued, hookworm disease experienced a significant decline in the southern United States that ultimately led to its elimination from the region (M 2009).

Although hookworm infection still exists in much of the developing world, this campaign demonstrated that a combination of education, infrastructure development and therapy can make a significant difference in the prevalence of hookworm infection.

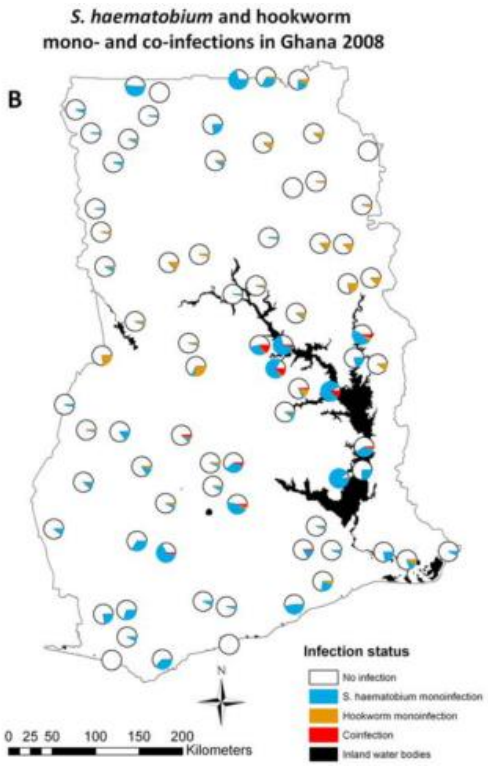
In an attempt to alleviate the disease burden in the developing world, the World Health Organization passed a resolution in 2001 at the 54th World Health Assembly to control infection (Humphries D 2012). This resolution urged "member states to provide regular drug treatment of high-risk groups," with the overarching goal of reaching at least 75% of school aged, at risk children by 2010 (Humphries D 2012). This resolution went on to recommend annual deworming in areas where the prevalence of STNs was higher than 20%.(Humphries D 2012) In an effort to control morbidity, the WHO launched a project to control soil transmission, which was first initiated in Asia (2004), East Africa (2005) and West Africa (2006) (Jiraanankul, Aphijirawat et al. 2011). As of 2013, the WHO continued to recommend annual or biannual treatment in endemic regions, with a focus on increasing

coverage in regions that did not meet coverage criteria. Alongside mass drug administration, improvements in water, sanitation, and socioeconomic status in Asia and Latin America have contributed to a decrease in the overall prevalence of hookworm infection (Brooker S 2004). However, rates of infection in sub-Saharan Africa continue to be high, as poor education, limited access to adequate health care, and unsanitary living conditions continue to provide conditions which favor transmission (Bungiro R 2011).

i. Previous Study in Ghana

Previous studies have found a high degree of variability of hookworm prevalence across Ghana. A cross-sectional study conducted throughout Ghana in 2011 found the prevalence of hookworm infection to be highly focal, with cases observed in mostly Central and Northern Ghana (Figure 2) (Soares Magalhaes, Biritwum et al. 2011).

Figure 3: Hookworm and Schistosome Mono- and Co-infections in Ghana Among Children ages 5-19



Soares Magalhaes, R. J., et al. (2011). "Mapping helminth co-infection and co-intensity: geostatistical prediction in ghana." PLoS Negl Trop Dis **5**(6): e1200.

A recent study conducted in Southern Ghana found a higher prevalence of infection in rural areas than in urban areas (13.6% vs. 0.1%), while a study conducted in the Volta region of Eastern Ghana found a 9.8% prevalence (van Mens, Aryeetey et al. 2013, Egbi, Steiner-Asiedu et al. 2014). In a study examining over 20,000 participants from 216 villages in Northern Ghana, all but one village (99.5%) had at least one case of hookworm present, with an overall prevalence of 50% (Yelifari, Bloch et al. 2005). Most studies found that participants were predominantly infected with *Necator americanus*, although *Anclystoma duodenale* was found in 20% of participants (de Gruijter, van Lieshout et al. 2005, Soares Magalhaes, Biritwum et al. 2011).

Previous research in Kintampo found a high prevalence of low intensity hookworm infections in Kintampo North prior to treatment (Humphries D 2012). Data from 2007 across four communities found a cure rate of 61% following administration of single dose albendazole therapy (Humphries D 2012). The fecal egg count reduction (FECR) rate was below the 90% mark for an effective therapy, at 82% (Humphries D 2012). A study of 16 schools in 13 communities in Kintampo in 2010 again found sub-optimal cure rate (44%) and FECR rate (87%) (Humphries D 2012). A 2011 study in five contiguous Kintampo communities again noted sub-optimal cure (37.2%) and FECR rate (60.4%) (Humphries D 2012). While the cure rate and FECR rate were relatively low in this study, albendazole response was highly variable across the five communities (Humphries D 2012). Most recently, study in Kintampo found a 39% prevalence of infection with a 43% cure rate and FECR rate of 87.3% after one dose of albendazole (Humphries D 2013). Given the moderately low cure rates and FECR rate observed in these studies, further investigation is warranted in order to ensure that resistance is observed as it arises.

j. Study Rationale and Objectives

The emergence of reduced albendazole efficacy for hookworm infection has major worldwide implications. We hypothesized that the decreasing effectiveness of albendazole therapy was due to reduced susceptibility of the parasite. This study provides important data about parasitic factors that may be contributing to a decrease in albendazole susceptibility. In order to evaluate this hypothesis, the primary objectives of this study were to:

1. Determine the baseline prevalence, intensity, and epidemiology of hookworm infection in four villages in Kintampo North Municipality. These villages (Jato, Cheranda, Mahama and Tahiru) previously exhibited high and low cure rates following albendazole therapy.
2. Determine the relationship between clinical response and in vitro albendazole susceptibility using human hookworm isolates.
3. Extract genomic DNA for future studies aimed at defining the molecular basis of albendazole resistance in Kintampo North.
4. Identify the species of hookworm from each positive sample

II. Methods

a. Ethical Approval and Informed Consent

The Yale University Human Investigation Committee (HIC) approved both field and laboratory components of this project in May, 2013 under protocol number 1304011926. Approval was also given by the Noguchi Memorial Institute for Medical Research (NMIMR) investigational review board (IRB), the Ghanaian Ministries of Health and Education, the Kintampo Health Research Centre (KHRC), the chiefs and elders of each community and the teachers and directions from each school.

Preliminary steps to begin recruiting participants began in May, 2013 when the research team initiated meetings to discuss the study protocol with directors at the Ministry of Health and Education and the Kintampo Health Research Center. After reviewing census data compiled in November 2012, it was determined that 226 children from 4 villages along the Techiman-Tamale highway north of Kintampo were eligible to be recruited for this study. Study subjects were included in the study if they: 1) resided within the study area, 2) were between the ages of 7-12, and 3) were willing and able to give consent. Children were excluded from the study if they were already enrolled in an ongoing longitudinal study conducted by colleagues at the NMIMR. Under the approved protocol, every child that met entry criteria would be asked to enroll in the study.

b. Study Site:

This study was conducted between May and July, 2013 along a 30km stretch of the Techiman-Tamale highway in Kintampo North Municipality, Ghana, between Tahiru (the southernmost village) and Cheranda (the northern most village) (Figure 1). Kintampo is part of the Brong Ahafo region of central Ghana, which predominantly agricultural, and cocoa, yam and cassava are frequently grown in this region. Farming is the most common

occupation, and sanitation and access to clean water are limited. Helminth infections are highly prevalent, especially among primary school children.

Figure 4: The Techiman-Tamale Highway in Kintampo North Municipality, Ghana

c. Study Participant Recruitment and Consent:

Meetings were initiated with local chiefs and elders in each village in order to discuss the study. Following approval, community meetings were set up in order to explain the purposes of the study to all interested parties in each village. All meeting were conducted by Ghanaian colleagues in the local language, Twi, to ensure that the study purposes were clearly understood. Following community meetings in each of the villages, potential study

participants were visited at their homes. Ghanaian colleagues reiterated the purposes of the study in Twi to children and their parents. For those that agreed to participate, written consent forms were signed by the child and their parent and a copy of the consent form was given to all enrolled participants for reference. After visiting the homes of all potential participants, the final study population consisted of 179 children ages 7-12 from 4 separate villages: Jato, Cheranda, Mahama and Tahiru.

d. Questionnaire

Upon enrollment into the study, the parent or guardian of each study participant was given a household questionnaire by two assistants from the Kintampo Health Research Center. This questionnaire contained questions that were included in a more extensive questionnaire conducted in January, 2013 when the community census took place. Interviews were conducted in Twi, and gathered information about the child's school and community, along with information about the household in which they lived. This assessment provided information about potential confounders including basic information about the child, age, socioeconomic status, and any environmental exposures that may have varied from household to household (Appendix 1).

e. Fecal Collection, Processing & Treatment:

Following consent, 500ml fecal collection cups were administered to each study participant. Children were instructed to fill the cups with fresh portions of their morning stool, and the following day, cups were collected from each child and taken back to the Kintampo Health Research Center in a cooler. The Kato-Katz technique was used in duplicate for each sample in order to identify a positive infection.(Katz N 1972) Laboratory personnel identified the presence or absence of parasite ova through the use of microscopy

and the number of eggs were counted on both slides and averaged. In order to calculate the total number of eggs per gram, the average was multiplied by a factor of 24. All samples that had at least one hookworm ova present were set aside for further processing. In addition to examining slides for hookworm ova, slides were counted for *Ascaris lumbricoides, Trichuris trichiura*, *Taenia spp.*, and *Hymenolepis nana* and counts were recorded for future reference.

A small amount of stool was set aside from each positive sample and pooled together by village. Each pool was used in the Baermann method (Appendix 3) in order to hatch hookworm ova and isolate hookworm larvae for future analysis. Hookworm ova were extracted from the remaining stool for each infected study participant (Appendix 2), and egg hatch assays were set up for samples that had a high enough number of eggs in order to measure pre-treatment susceptibility to albendazole (Appendix 4). The remaining extracted eggs were frozen in the lab for future analysis.

Each child that was positive for hookworm was treated with a single dose of 400mg albendazole within a week of laboratory diagnosis. Ten to fourteen days after treatment, a second stool sample was collected and the Kato-Katz technique was again repeated in order to identify positive infections. Egg extractions, Egg Hatch Assays and the Baermann method were conducted for all positive post-treatment samples and eggs were frozen for future analysis. Children that were infected with hookworm post-treatment were given an additional dose of 400mg albendazole within a week of identification. At this time, children with other parasitic infections were also treated with albendazole (*T. trichiura*) or praziquantel (*H. nana*).

f. Molecular Methods:

Following the completion of post-treatment collections, genomic DNA was extracted at the Kintampo Health Research Center from all pre- and post-treatment eggs using the QIAamp DNA stool Kit (Qiagen). Larvae samples were pelleted down, and both extracted

DNA from eggs and pelleted larvae were taken back to the Cappello laboratory for future analysis.

Starting in September of 2013, the Cox-1 polymerase chain reaction (PCR) protocol was used in order to determine which species of hookworm (*Necator americanus* or *Ancylostoma duodenale*) was present in samples. PCR products for both NA and AD were used in gel electrophoresis, and those with visible product were extracted in order to obtain purified DNA. Purified DNA was combined with primer and TBE buffer and sent to a lab at the Yale School of Medicine for sequencing. Sequences were returned for each product and BLASTed on the NCBI database. This was done in order to see if sequences matched other *Necator americanus* or *Ancylostoma duodenale* isolates within the database. Those that were matches were noted as confirmed cases of either subtype.

g. Data Analysis:

There are three measurements that were calculated to access drug effectiveness: the fecal egg count reduction rate (FECR), the cure rate (CR), and the hatch rate (HR) (Vercruysse J 2011). While the cure rate tells us about the change in the prevalence of the disease, the egg reduction rate tells us the percentage decrease from baseline to post-treatment levels (Anantaphruti M 2007). The hatch rate calculates in vitro susceptibility of hookworm eggs to albendazole.

$$\text{FECR} = \frac{\text{Mean EPG } before \text{ deworming} - \text{Mean EPG } after \text{ deworming}}{\text{Mean EPG } before \text{ deworming}} * 100\%$$

$$\text{CR} = \frac{\%\text{ Prevalence } before \text{ treatment} - \%\text{ Prevalence } after \text{ treatment}}{\%\text{ Prevalence } before \text{ treatment}} * 100\%$$

$$\text{HR} = \frac{\text{number of hatched larvae}}{\text{number of hatched larvae} + \text{number of remaining eggs}} * 100\%$$

At the conclusion of the study, the CR and FECR were compared between the two villages. Those individuals that were still excreting eggs in their stool (treatment failures)

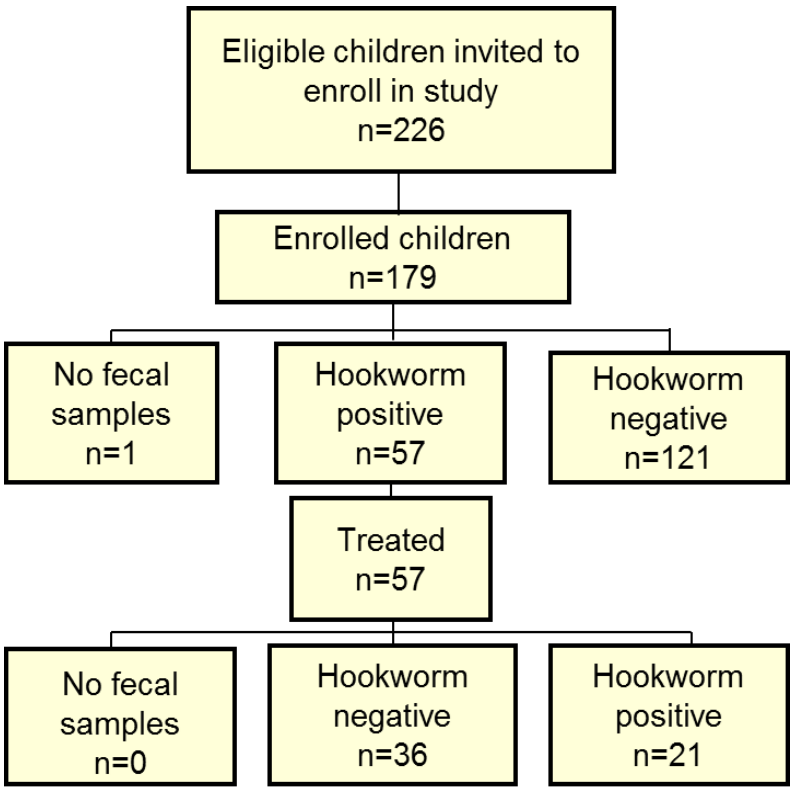
were given another dose of 400 mg albendazole. Only study subjects with complete data sets were included in the analysis stage. Data was stored in Microsoft Excel, and statistical analysis was conducted with SAS version 9.3. Descriptive statistics detailing demographics, socioeconomic status, nutritional status, and hookworm infection intensity were also calculated for the two groups of children. Univariate statistical analyses of pre- and post-treatment study populations will be used for descriptive purposes. The Chi-Squared test was used to examine differences between populations and groups, while T-tests were used for continuous variables. Unadjusted logistic regression was used to identify explanatory variables, which were then used in multivariate analysis. This statistical technique allowed for the assessment of any factors that could be mediating decreased albendazole susceptibility.

III. Results

a. Study Population:

At the time of enrollment, 226 children were eligible in the four communities to enroll in the study. Of these, 179 were enrolled in the study and fecal samples were received from a total of 178 children. Fifty-seven children were hookworm positive after baseline collections, while 121 children were negative for hookworm infection. All 57 children that were hookworm positive at baseline were treated with a single dose of 400mg albendazole. Post-treatment (10-14 days) samples were received from all 57 children. Of these, 36 children fully responded to treatment and were hookworm negative, while 21 children were still positive for hookworm infection.

Figure 5: Characteristics of Study Population



b. Baseline Characteristics of Study Population:

Table 1. Demographic and Socioeconomic Indicators of the Study Population at Baseline

Characteristic	**Study Population (N = 178)**[b]
Age (years)	9.45 ± 1.72
Sex	
Female	50 (89)
Male	50 (89)
Body Mass Index	16.75 ± 4.1
Average Household Size	11.54 ± 6.94
Absolute Wealth	
Low	33.15 (59)
Middle	42.70 (76)
High	24.16 (43)
Ownership of agricultural land	
Yes	81.46 (145)
No	18.54 (33)
Household savings account	
Yes	52.25 (93)
No	47.75 (85)
Daily use of a Latrine or Toilet	
Yes	21.91 (39)
No	78.09 (139)
Primary Religion	
Muslim	12.92 (23)
Christian	70.22 (125)
Traditional	12.36 (22)
Other	4.49 (8)
Attends School	
Yes	95.51 (170)
No	4.49 (8)
Daily Shoe Usage	
Yes	82.58 (147)
No	0.17 (31)

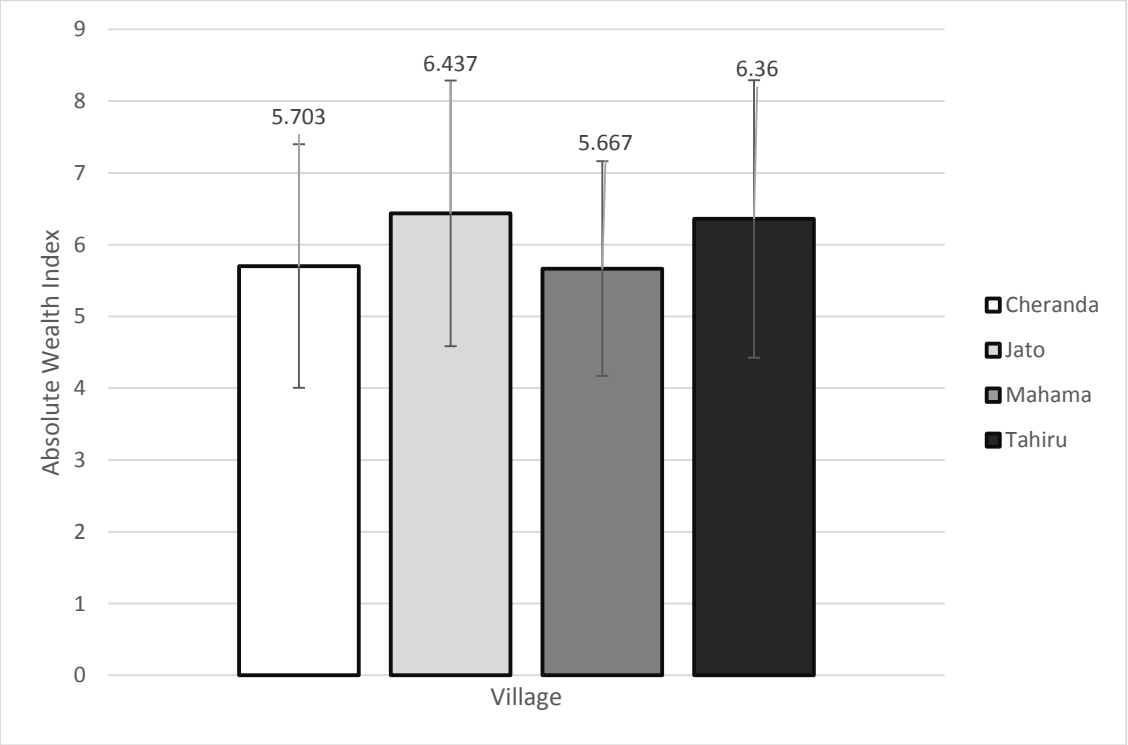
[a]Table values are column % (N) for categorical variables and mean ± SD for continuous variables
[b]Numbers may not sum to total due to missing data and percentages may not sum to 100% due to rounding

Initial analysis of the baseline population was done with univariate statistics in SAS v. 9.3. The average age of children enrolled (n=178) was 9.5 years old. Eighty-nine (50%) study participants were male while 89 (50%) were female. Children had an average body mass index of 16.75 and only 8 children (4.49%) did not attend school. There were approximately 12 people per household, and the majority of households owned agricultural land (81.5%)

and were Christian (70.22%). Most children had daily access to a toilet or latrine (78.09%) and wore shoes daily (82.58%).

An absolute wealth index was calculated using data from 18 measurements in order to estimate the approximate wealth of each household.(Filmer and Pritchett 2001) This measure of socioeconomic status included a variety of variables from the household questionnaire (ownership of pigs, poultry, goats or sheep, horses or donkeys, a tile floor, use of advanced cooking fuel, electricity, radio, TV, phone, refrigerator, bike, car or motorcycle, ownership of land,a bank or savings account, improved water source, and an improved toilet) in order to compare absolute wealth at the community level.(Filmer and Pritchett 2001, Humphries D 2013) The mean absolute wealth index across the study was 6.08 with a standard deviation of 2.065. Children from Mahama had the lowest absolute wealth index (5.667), while children in Jato had the highest absolute wealth index (6.437).

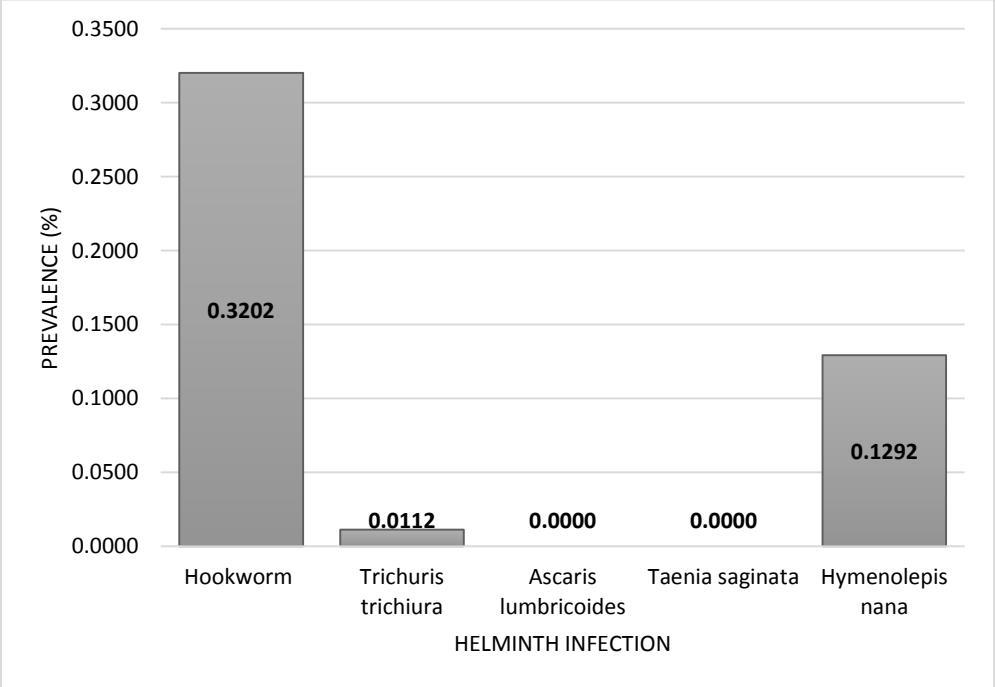
Figure 6: Absolute Wealth Index by Village



c. Prevalence of Intestinal Helminth Infections at Baseline:

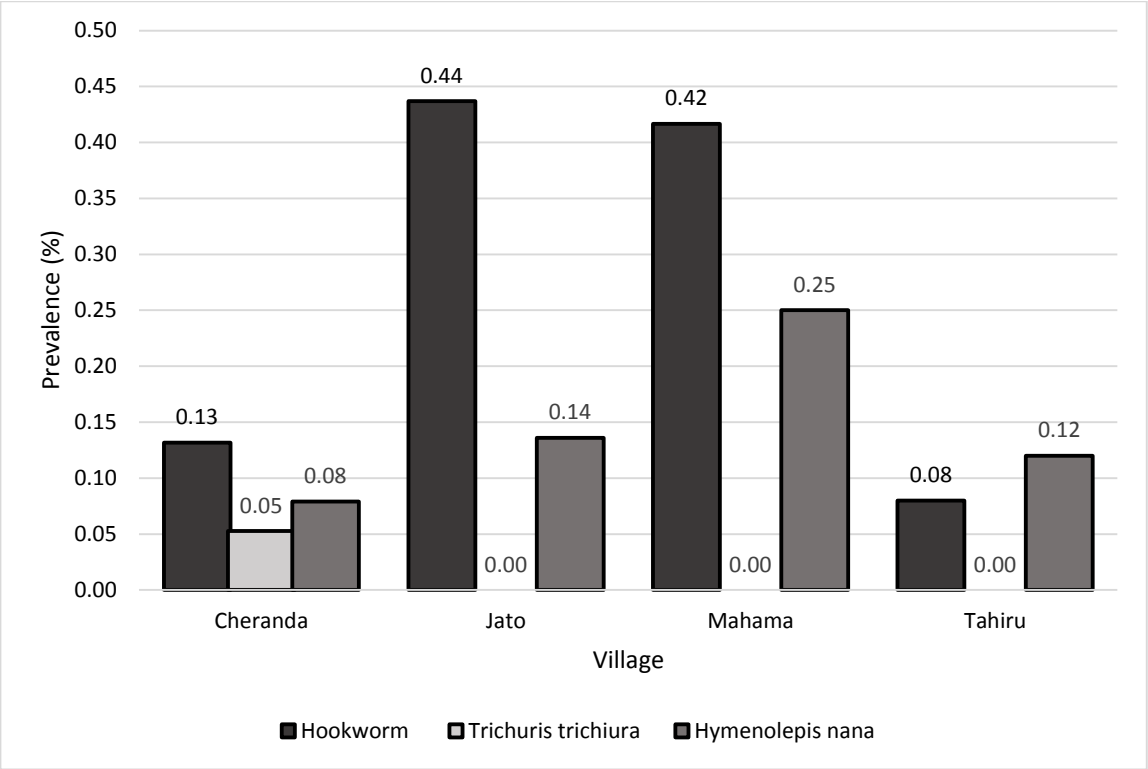
After screening, the highest prevalence of Helminth infection was hookworm infection, which occurred in 32.02% of study participants (n=57) (Figure 6). *Trichuris trichiura* was observed among 1.12% of study participants (n=2) while *Hymenolepis nana* was found among 12.92% (n=23) study participants (Figure 6). Although fecal smears were examined for *Ascaris lumbricoides* and *Taenia saginata*, no cases were detected among study participants at baseline.

Figure 7: Prevalence of Intestinal Helminth Infections at Baseline



Hookworm infection was found in all four communities (figure 7) with varying levels of infection. *T. trichiura* was observed only in Cheranda, while *H. nana* was observed across all four communities, with the highest prevalence in Mahama, where 25% of study participants were infected. No cases of *A lumbricoides* or *T. saginata* were observed among study participants at baseline.

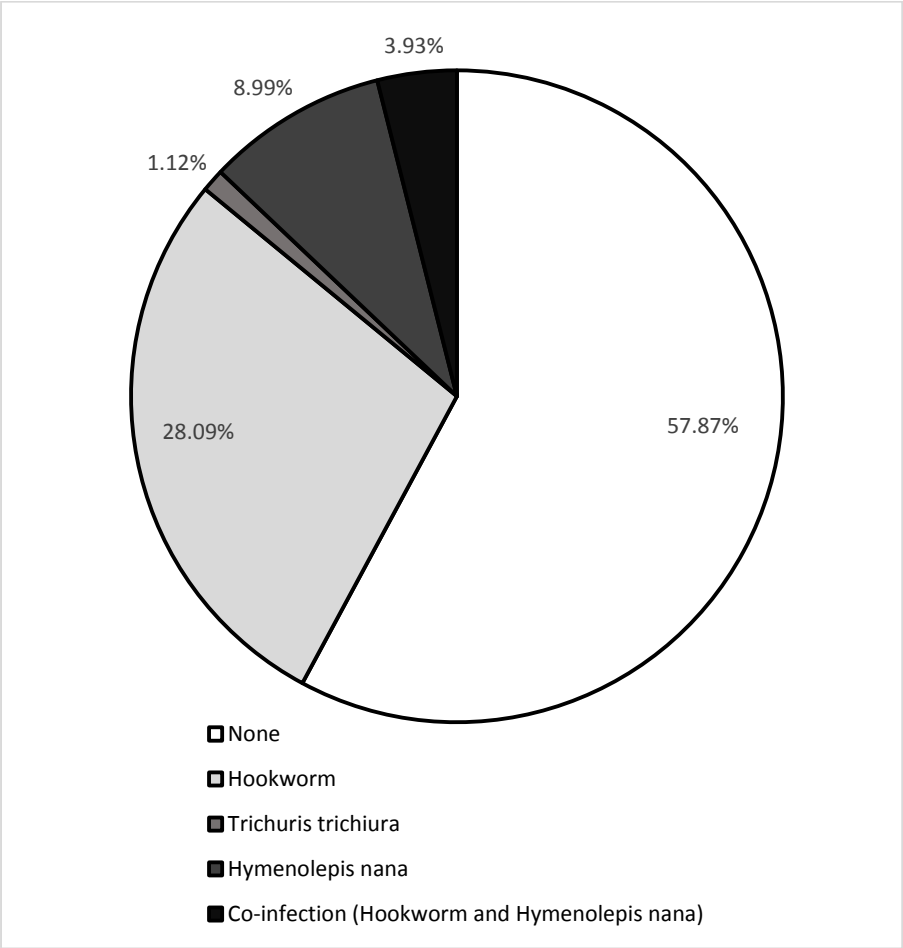
Figure 8: Prevalence of Intestinal Helminth Infections at Baseline by Village[a]



[a]*Ascaris lumbricoide* and *Taenia saginata* excluded due to 0% prevalence among study participants

Out of a a possible 178 study participants, 103 (57.87%) had no visible helminth eggs of any species in their stool (Figure 8). Few (1.12%) subjects were positive for *T. trichiura*, while 8.99% (n=16) of participants were positive for only *H. nana* (Figure 8). Isolated hookworm monoinfection was observd in 23.09% of participants (n=50), while 3.93% (n=7) of participants were co-infected with hookworm and *H. nana*.

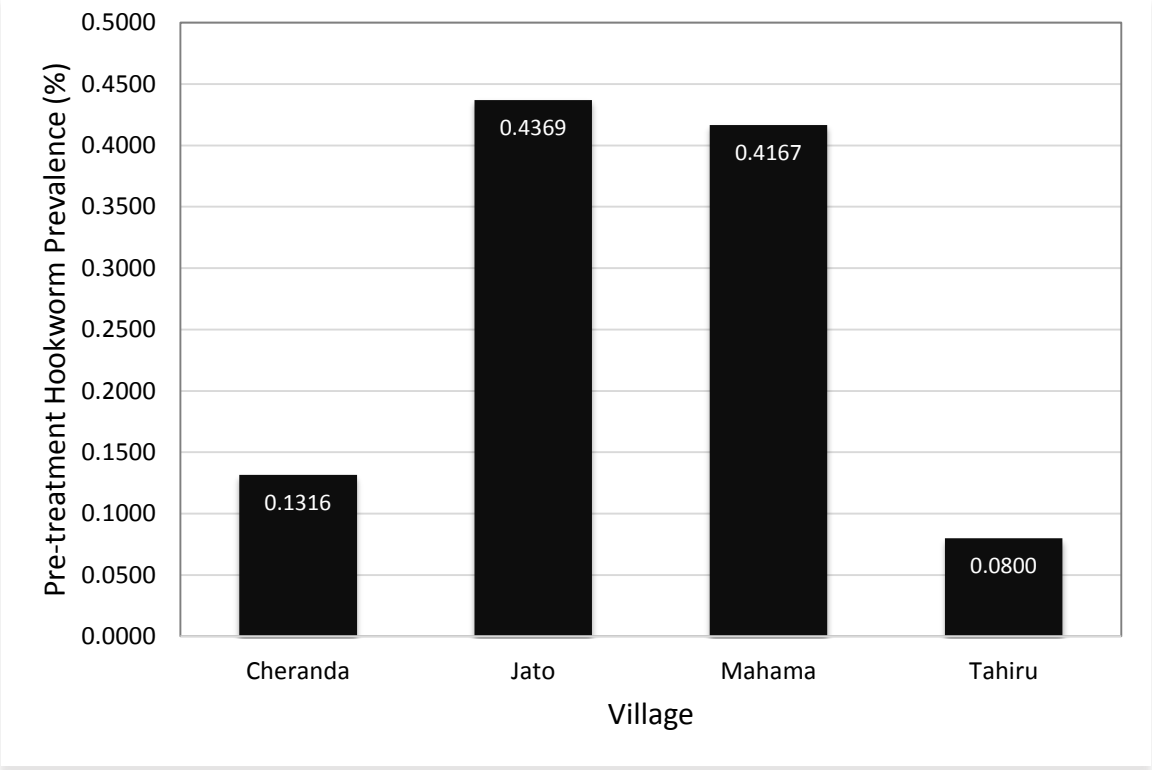
Figure 9: Intestinal Helminth Mono- and Co-infection at Baseline (n=178)



d. *Prevalence & Intensity of Hookworm Infection at Baseline:*

After collecting initial baseline stool samples from each of the study participants, a total of 57 children from all four communities were positive for hookworm. While 3.51% of hookworm positive cases were from Tahiru (N=2), 8.77% of cases (N=5) each came from Mahama and Cheranda, and 78.95% of cases (N=45) were from Jato (Figure 3). Although the highest number of cases came from Jato, both Jato and Mahama had high prevalences of hookworm (43.69% and 42.67% respectively) while Tahiru (8.00%) had the lowest prevalence (Figure 3).

Figure 10: Pre-treatment Hookworm Prevalence by Village



Almost all cases (98.25%) were characterized as having a light infection (Table 2). Only one child from Jato was noted to have a heavy infection of over 4000 eggs per gram (EPG). Children that were infected at baseline had a mean egg count of 443epg, and egg counts ranged from 12-6336epg among the sample of children positive at baseline (Table 2).

Table 2. Intensity of Hookworm Infection at Baseline

Classification[a]	**N (%)**[b]
Light (1-1,999 Eggs per gram)	56 (98.25)
Moderate (2,000-3,999 Eggs per gram)	0 (0)
Heavy (>4000 Eggs per gram)	1 (1.75)

[a]Based on WHO criteria (Pawlowski Z 1991)

e. *Demographic and Socioeconomic Analysis of the Baseline Study Population by Infection Status*

After collecting and processing all stool samples, univariate statistics were computed for a variety of variables based on hookworm infection at baseline. Continuous variables were analyzed using t-tests while chi-squared tests were used for categorical and binary variables. Children that were positive at baseline were slightly younger (9.13 years old) than those that were uninfected (9.69 years old), although this was not statistically significant ($p=0.10$). Gender, Body Mass Index (BMI), Pig Ownership, Land ownership and Absolute Wealth index were also non-significant between hookworm positive and hookworm negative subgroups ($p=0.6298$, 0.8562, 0.6494, 0.2885 and 0.2629 respectively). Religion was found to be significant ($p=0.0278$) and Christians and Traditionalists had the highest number of positives. Tribal identity was highly significant ($p<0.0001$), with almost all positive cases from the Konkomba and Chokose tribes, and no cases among the Mo tribe. The average household size was also significantly higher among children that were infected at baseline as compared to children that were uninfected ($p=0.002$). There was higher prevalence of infection among children who did not attend school at baseline ($p=0.008$) (Table 3).

Table 3. Demographic and Socioeconomic Indicators of the Study Population by Hookworm Infection Status at Baseline[a]

	Hookworm Infection		
Characteristic	**% Positive (N = 57)[b]**	**% Negative (N = 121)[b]**	**p[c]**
Age (years)	9.13 ± 1.71	9.69 ± 1.71	0.1000
Sex			0.6298
Female	47.37 (27)	51.24 (62)	
Male	52.63 (30)	48.76 (59)	
Body Mass Index	16.65 ± 5.97	16.80 ± 2.85	0.8561
Village			0.0002
Cheranda	8.77 (5)	27.27 (33)	
Jato	78.95 (45)	47.93 (58)	
Mahama	8.77 (5)	5.79 (7)	
Tahiru	3.51 (2)	19.01 (23)	
Tribe			0.0001
Konkomba	68.42 (39)	53.72 (65)	
Mo	0.00 (0)	17.36 (21)	
Chokose	29.82 (17)	15.70 (19)	
Other	1.75 (1)	13.22 (16)	
Absolute Wealth Index			0.2629
Low	28.07 (16)	35.54 (43)	
Medium	40.35 (23)	43.80 (53)	
High	31.58 (18)	20.66 (25)	
Primary Religion			0.0278
Muslim	5.26 (3)	16.53 (20)	
Christian	70.18 (40)	70.25 (85)	
Traditional	21.05 (12)	8.26 (10)	
Other	3.51 (2)	4.96 (6)	
Attends School			0.0077
Yes	89.47 (51)	98.35 (119)	
No	10.53 (6)	1.65 (2)	
Average household size	13.82±6.78	10.48±7.82	0.0023
Ownership of Land			0.2885
Yes	85.96 (49)	79.34 (96)	
No	14.04 (8)	20.66 (25)	
Ownership of Pigs			0.6494
Yes	19.30 (11)	16.53 (20)	
No	80.70 (46)	83.47 (101)	

[a]Table values are mean ± SD for continuous variables and column % (N) for categorical variables

[b]Numbers may not sum to total due to missing data and percentages may not sum to 100% due to rounding

[c]p-value is for t-test (continuous variables) or x^2-test (categorical variables)

Daily use of a toilet or latrine was also found to be highly statistically significant (<0.0001), with only one case among children that used a toilet or latrine daily (Table 4). Infection with another helminth infection (*Trichuris* or *H. nana*), daily shoe usage, and antiparasitic treatment in the last year were not found to be significantly associated with hookworm infection (p=0.6420, 0.3799, 0.8795 respectively).

Table 4. Host Factors of the Study Population by Hookworm Infection Status at Baseline[a]

	Hookworm Infection		
Characteristic	**% Positive (N = 57)[b]**	**% Negative (N = 121)[b]**	**p[c]**
Infection with another Helminth			0.6420
Yes	12.28 (7)	14.88 (18)	
No	87.72 (50)	85.12 (103)	
Daily Shoe Usage			0.3799
Yes	78.95 (45)	84.30 (102)	
No	21.05 (12)	15.70 (19)	
Daily Toilet or Latrine Use			<0.0001
Yes	1.79 (1)	31.40 (38)	
No	98.25 (56)	68.60 (83)	
Antiparasitic treatment in the last year			0.8795
Yes	24.56 (14)	25.62 (31)	
No	75.44 (43)	74.38 (90)	

[a]Table values are mean $\pm$ SD for continuous variables and column % (N) for categorical variables

[b]Numbers may not sum to total due to missing data and percentages may not sum to 100% due to rounding

[c]p-value is for t-test (continuous variables) or x^2-test (categorical variables)

f. Risk Factors for Hookworm Infection at Baseline

Using the risk factors for infection, unadjusted logistic regression was used to identify variables for use in multivariate regression analysis. Odds ratios were adjusted for age, religion, body mass index (BMI), absolute wealth index, land ownership, infection with another helminth, and antiparasitic treatment in the last year. School attendance and daily toilet or latrine use were found to be risk factors for hookworm infection (p=0.0137 and 0.0482 respectively), as was tribe. Specifically, being a member of the Konkomba or Chokose tribe was associated with an increased risk of having hookworm infection (p=0.0470 and 0.0439 respectively), while being a member of the Mo tribe was highly non-significant (p=0.9685) (Table 5).

Table 5. Risk Factors for Hookworm Infection at Baseline[a]

Characteristic	Adjusted Odds Ratio	95% Confidence Interval	p[c]
Male	0.863	0.391-1.906	0.7163
Village (reference=Tahiru)	1.00		
Cheranda	0.780	0.020-30.680	0.8942
Jato	0.356	0.013-9.597	0.5389
Mahama	0.857	0.031-23.420	0.9270
Tribe (Reference=other)	1.00		
Konkomba	28.090	1.045-755.150	0.0470
Mo	34.006	1.102->999	0.0439
Chokose	<0.001	<0.001->999	0.9685
Household size	1.053	0.993-1.117	0.0829
Pig Ownership	0.635	0.209-1.930	0.4230
Daily Shoe Usage	1.881	0.684-5.176	0.2209
Attends School	0.034	0.002-0.500	0.0137
Daily Toilet or Latrine Use	0.011	<0.001-0.965	0.0482

[a]Adjusted for all other variables in this model

g. Post-Treatment Demographic and Socioeconomic Assessment Based on Treatment Response

Table 6. Demographic and Socioeconomic Indicators of the Treatment Population by Post-Treatment Hookworm Infection Status[a]

	Hookworm Infection		
Characteristic	**% Positive (N = 21)[b]**	**% Negative (N = 36)[b]**	**p[c]**
Age (years)	8.95 +/- 1.82	9.38 +/- 1.71	0.3829
Sex			0.9769
Female	47.37 (10)	47.22(17)	
Male	52.38 (19)	52.78 (19)	
Body Mass Index	15.82+/- 1.83	17.13 +/- 7.38	0.3183
Village			0.1212
Cheranda	0.00 (0)	13.89 (5)	
Jato	95.24 (20)	69.44 (25)	
Mahama	1.75 (1)	11.11 (4)	
Tahiru	0.00 (0)	5.56 (2)	
Tribe			0.6923
Konkomba	66.67 (14)	69.44 (25)	
Chokose	0.00 (0)	0.00 (0)	
Mo	33.33 (7)	27.78 (10)	
Other	0.00 (0)	2.78 (1)	
Absolute Wealth Index			0.7914
Low	28.57 (6)	27.78 (10)	
Medium	33.33 (7)	41.67 (15)	
High	38.10 (8)	30.56 (11)	
Primary Religion			0.4965
Muslim	9.52 (2)	2.78 (1)	
Christian	71.43 (15)	69.44 (25)	
Traditional	19.05 (4)	22.22 (8)	
Other	0.00 (0)	5.56 (2)	
Attends School			0.2788
Yes	95.24 (20)	86.11 (31)	
No	4.76 (1)	13.89 (5)	
Average Household Size	12.76 ± 6.76	14.44 ±8.41	0.4384
Ownership of Land			0.2345
Yes	80.95 (17)	91.67 (33)	
No	19.05 (4)	8.33 (2)	
Ownership of Pigs			0.1532
Yes	9.52 (2)	25.00 (9)	
No	90.48 (19)	75.00 (27)	

[a]Table values are mean ± SD for continuous variables and column % (N) for categorical variables

[b]Numbers may not sum to total due to missing data and percentages may not sum to 100% due to rounding

[c]p-value is for t-test (continuous variables) or x^2-test (categorical variables)

Table 7. Host Indicators of the Treatment Population by Post-Treatment Hookworm Infection Status[a]

	Hookworm Infection		
Characteristic	**% Positive (N = 21)[b]**	**% Negative (N = 36)[b]**	**p[c]**
Infection with Another Helminth			0.1152
Yes	14.29 (3)	47.22(17)	
No	85.71 (18)	66.67 (24)	
Daily Shoe Usage			0.6966
Yes	76.19 (16)	80.56 (29)	
No	23.81 (5)	19.44 (7)	
Daily Toilet or Latrine Usage			0.4410
Yes	21 (100.00)	2.78 (1)	
No	0 (0.00)	97.22 (35)	
Antiparasitic Treatment in the last year			0.5911
Yes	28.57 (6)	22.22 (8)	
No	71.43 (15)	77.78 (28)	

[a]Table values are mean $\pm$ SD for continuous variables and column % (N) for categorical variables

[b]Numbers may not sum to total due to missing data and percentages may not sum to 100% due to rounding

[c]p-value is for t-test (continuous variables) or x^2-test (categorical variables)

Post-treatment univariate analysis was conducted among children that received treatment and submitted a second fecal sample. This analysis was divided between children that were positive and children that were negative post-treatment, and no variables were found to be significantly associated with post-treatment response (Table 6 and 7).

h. *Cure Rate and Fecal Egg Reduction Rate*

Following examination of post-treatment isolates, the overall cure rate among infected children (n=57) was 63.2% (n=36) (Figure 11). The highest cure rates were observed in both Tahiru and Cheranda (100%), Mahama had a cure rate of 80% and the lowest cure rate was observed in Jato, where only 55% of children were cleared of infection following a single dose of the drug.

Figure 11: Community Variation in Response to Treatment

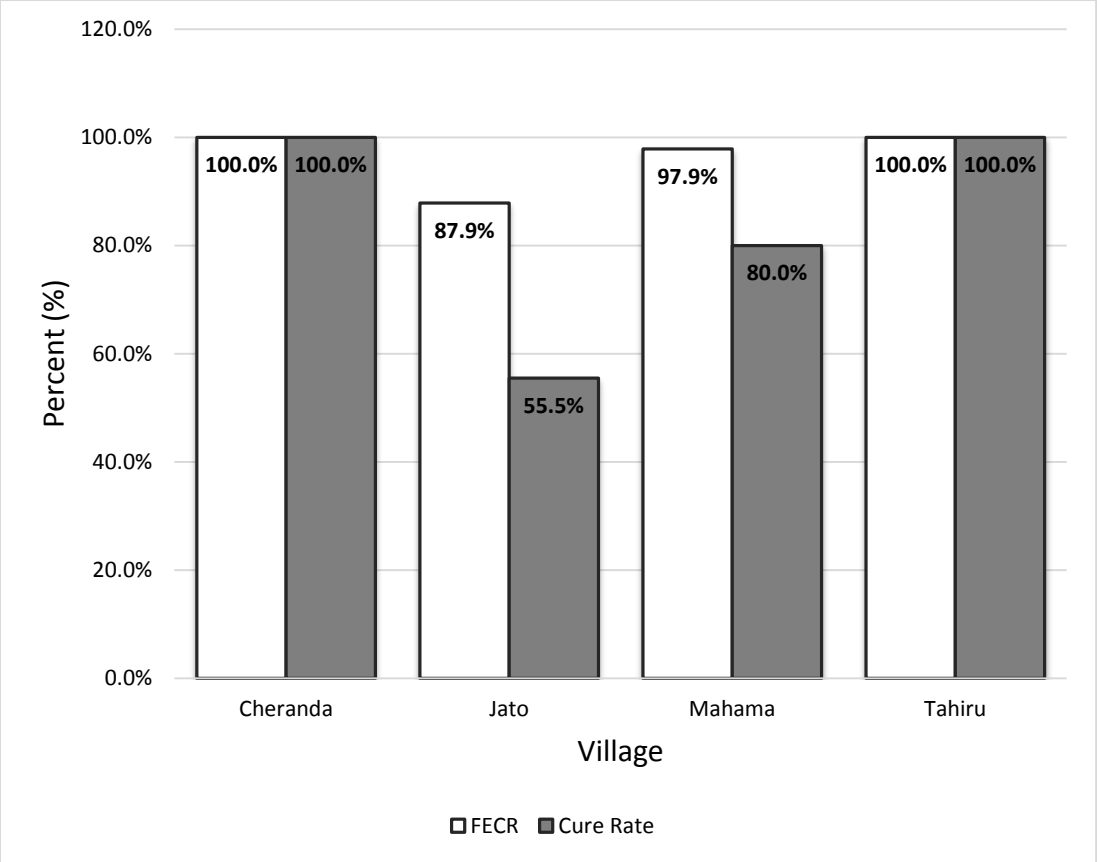


Table 8. Hookworm Cure Rate and Egg Reduction Rate of the Treatment Group	
Prevalence (%)	
Baseline	100
Post-Treatment	36.84
Cure Rate (%)	63.2
Arithmetic Mean Egg Count[a]	
Baseline	442.53 ± 909.19
Post-Treatment	112.00± 191.97
Egg Reduction Rate (%)	90.7

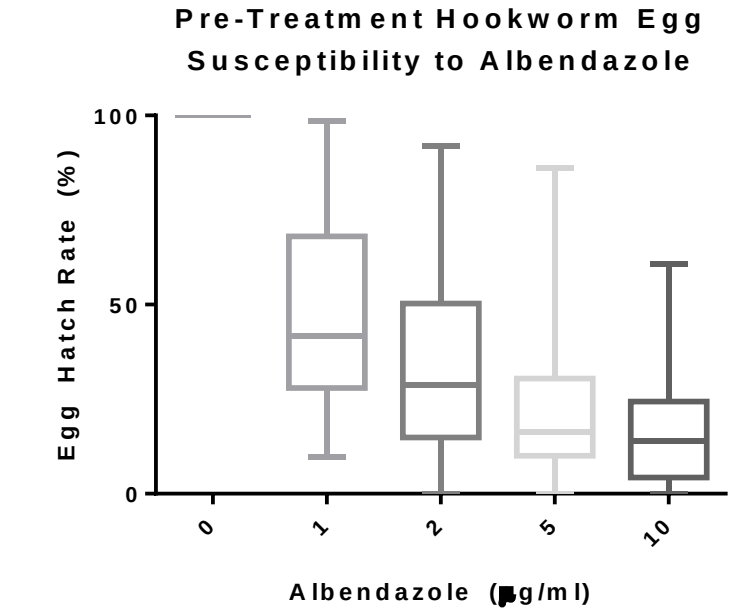
[a]Eggs per gram

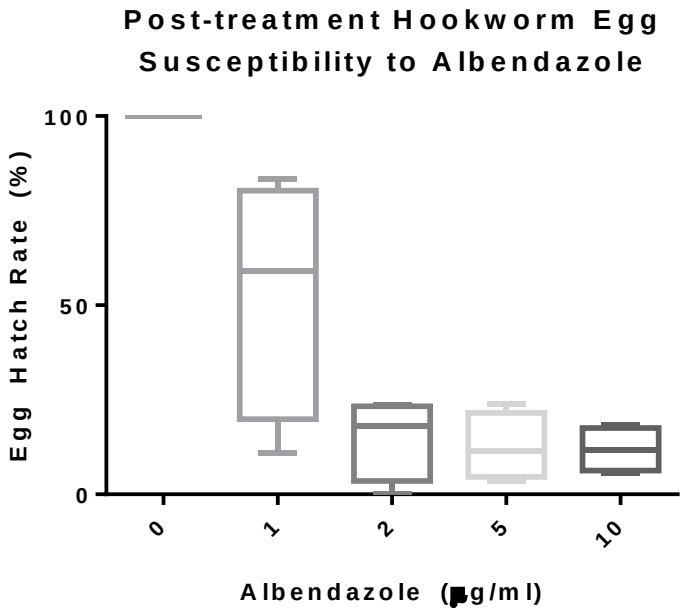
Children at baseline had a higher mean egg count (442.53epg) than children that were positive following a single dose of albendazole (112epg) (Table 8). There was also more variation in egg count among children that were positive at baseline (standard deviation +/- 909epg) and children that were positive post-treatment (191.97). All children that were positive post treatment (n=21) had a light infection (less than 1,999 eggs per gram).

i. In Vitro *Susceptibility of Hookworm Isolates*

Egg hatch assays (EHA) were set up for samples that had counts greater than 10 eggs/30µl, and incubated at room temperature for 24 hours. 20 pre-treatment samples and 4 post-treatment samples had high enough egg counts to be used in the EHA. Figure 12 shows that for both pre- and post-treatment samples, as the concentration of albendazole increases, fewer eggs hatch. The median egg hatch rate at the highest concentration was similar at concentrations of 2, 5 and 10 µg/ml, and higher post-treatment at 1 µg/ml. However, these comparisons are limited due to the very low number of EHA data from post-treatment samples.

Figure 12: Susceptibility of Hookworm eggs to Albendazole at Increasing Concentrations Before and After Treatment





j. Identification of Hookworm Species using PCR:

After setting up PCRs for all pre-treatment samples, a total of 39 yielded product during gel electrophoresis. After extracting all bands from the gel and submitting the purified products for sequencing, a 15/16 of the samples submitted for *Necator americanus* were confirmed through sequencing, while 0/23 products were confirmed for *Ancylostoma duodenale.* Based on these results, all confirmed isolates were positive for *Necator americanus,* and there were no confirmed *Ancylostoma duodenale* positive sequences.

IV. Discussion

Epidemiology of Hookworm Infection

Soil-transmitted nematodes (STNs) including *Ascaris lumbricoides, Trichuris trichiura,* and hookworms are estimated to infect one out of every two people in developing countries (Humphries D 2012). Hookworm is a STN estimated to infect 740 people around the world, with the highest prevalence in sub-Saharan Africa (29%), where nearly 200 million people are infected (Humphries D 2012). STN infections have been targeted by a variety of control measures, including but not limited to health education, the introduction of latrines and better sanitary practices and chemotherapy (Albonico A, 1997). In recent years, the World Health Organization has taken an active role in trying to lower the disease burden and DALYs associated with STN infections through the use of mass drug administration (MDA). Currently, the World Health Organization (WHO) recommends annual deworming in areas with more than a 20% prevalence of STN infection and bi-annual treatment to regions where prevalence is 50% or higher. Worldwide, a single dose of albendazole is one of the recommended treatments for hookworm infection.

In Ghana, the prevalence of STN infections ranges from 40% in the central region to 87% in the north (Humphries D 2012). Previous study on hookworm infection in Kintampo, Ghana reported a prevalence of 45% in 2007, 39% in 2010, and 57% in 2011 (Humphries D 2012). This study found a lower prevalence of hookworm infection (32%) among the study population than in previous years. Similar to previous study in Kintampo, nearly every child (98.25%) harbored a light infection (1-1,999 eggs per gram) at baseline, and all children were lightly infected after treatment.

Previous study in Kintampo found other intestinal parasites including *Hymenolepis nana* and *Taenia spp.* to infect 15% of the study population. Notably, *Ascaris lumbricoides* and *Trichuris trichiura* were not observed among any participants. This study found both *H.*

nana (12.92%) and *T. trichiura* (1.12%) to be present in the study population, while *A. lumbricoides* and *Taenia spp.* were absent.

Human hookworm infection is primarily caused by two species of hookworm, *Necator americanus* and *Anclystoma duodenale*.(van Mens, Aryeetey et al. 2013) Infection with hookworms is endemic in many countries across sub-Saharan Africa. In Ghana, *N. americanus* is considered to be the predominant species, although studies have found that *A. duodenale* can coexist (de Gruijter, van Lieshout et al. 2005, van Mens, Aryeetey et al. 2013). Previous study in Kintampo in 2010 found most children to be infected with *N. americanus* (91.2%) (Humphries D 2013). Coinfection with *A. duodenale* to occur in a few cases (1.9%), and no isolated *A. duodenale* infections were observed during this study (Humphries D 2013). This study reaffirmed previous studies, finding 16/16 sequences to be positive for *Necator americanus* following DNA extractions and PCR. Although no *Ancylostoma duodenale* were observed in isolates, this may be due to the low number of successful isolates sequenced (16/78), and further analysis of these samples is warranted.

Risk Factors for Infection

Given its association with rural poverty, risk factors for hookworm infection are consistently reported to be related to socioeconomic status. Limited access to clean water, sanitation and adequate health care, along with poor education provide an environment around which transmission can continue. Previous study in Kintampo identified education level, occupation, and malaria parasitemia to be risk factors for infection in 2007. A 2010 study in Kintampo found pig ownership, parental occupation (farmer), larger household sizes, and not seeing a health care provider in the last year to be significantly associated with infection at baseline. This study found religion, village, tribal identity, not attending school, a larger household size, and not using a toilet or latrine daily to be statistically significant at the

0.05 level, indicating an association with helminth infection at baseline. Both school attendance and a larger household size are consistent with previous findings in Kintampo. Notably, this study was the first in Kintampo to identify religion, tribe and not using a toilet or latrine daily to be significantly associated with hookworm infection at baseline.

Following analysis with Multivariate Logistic Regression, risk factors for infection at baseline were identified. It was found that children that attended school had an Odds Ratio (OR) of 0.03 (p=0.01), indicating that school attendance was protective for infection with hookworm. This finding is consistent with previous study in Kintampo, which identified a higher education level as a protective factor for infection. Having daily access to a latrine was associated with a decreased risk for infection (OR=0.01), and children that had daily access were approximately ten times less likely to be infected than children that did not have daily access (p=0.049). While this was not found previously in Kintampo, lack of access to latrines has been found to be a risk factor for infection in previous studies (Chongsuvivatwong, Pas-Ong et al. 1996, Olsen, Samuelsen et al. 2001).

This was the first time that questions about tribal identity were asked to study participants in Kintampo. Findings demonstrated that being a member of the Konkomba or Mo tribes was associated with infection at baseline (p=0.047 and 0.044 respectively), while being a member of the Mo tribe was highly protective, with no cases of hookworm at baseline (p=0.969).

Treatment Efficacy

A single dose of albendazole (400mg) is frequently used in mass drug administration (MDA) campaigns as the primary drug recommended for the treatment of hookworm infection. Studies have found cure rates following administration to range from 33-95% and

the fecal egg count reduction rate to range from 64% to 100% (Keiser J 2010, Humphries D 2013, Samuel, Degarege et al. 2014).

Previous study in Kintampo found that cure rates (CR) after administration of a single dose of albendazole were shown to decrease over time, as 61% of the study population treated at baseline cleared infection in 2007, 43% cleared infection in 2010, and 39.5% cleared infection in 2011 (Humphries D 2012). Similarly, the mean fecal egg count reduction rate (FECR) fell from 81.5% in 2007 to 70.9% by 2011. This study found a higher cure rate (63.2%) and fecal egg count reduction rate (90.7%) than in previous years. While these data show the drug to be more effective than previously thought, there was a large degree of variation in treatment response at the community level.

Data from 2011 indicated that there was large variation in treatment response between villages. None of the treated children from Jato were cleared of infection after treatment and the mean FECR among children treated was only 1.2% (Humphries D 2012). In comparison, the cure rate in neighboring village Cheranda was 81.8%, with a mean FECR of 96.3% (Humphries D 2012). This study was conducted as a follow-up in order to further examine these differences in treatment response.

During previous years, a sample of children were enrolled from each community to provide an estimate of the disease burden in each community. This study enrolled every available child from both Jato and Cheranda, along with children from Mahama and Tahiru. The prevalence of infection varied by community, with 45% of children positive for hookworm in Jato, and 8.0% of children positive in Tahiru. The Fecal Egg Reduction Rates (FECR) and Cure rates (CR) were highest in Cheranda and Tahiru (100%) and lowest in Jato, where only 55% of children were cured of infection, and 87.5% of children experience a reduction in their egg counts. As was seen in Cheranda, Mahama, and Tahiru, nearly every child responded to therapy and was cleared of infection after a single dose of the drug (CR=

91.67%). In Jato, a village that neighbors both Cheranda and Mahama, 44.44% of children were positive for infection after treatment (CR=55.56%). Together, these data provide evidence that the effectiveness of albendazole treatment varies widely at the community level. These findings are consistent with data from the 2011 field study in Jato and Cheranda, which similarly observed differences in treatment response by community.

While these findings demonstrate a wide degree of variability in baseline prevalence and treatment response between villages, it is important to note that each community is not independent. Interestingly, all five cases of hookworm in Cheranda were in homes that were on the outskirts of town, nearest Jato. While this study did not take GPS coordinates of each home, the fact that each positive case from Cheranda came from locations nearest Jato is important to keep in mind.

Study Limitations

Limitations of this study include the use of the Kato-Katz technique to diagnose infection. The Kato-Katz is recommended by the WHO as a useful technique that provides an indirect measure of worm burden (Montresor A. 1998). However, its use is expected to underestimate the disease burden within a community (Montresor A. 1998). This is especially problematic for samples that have been left out, as hookworm eggs rapidly deteriorate in stool (Montresor A. 1998). This deterioration could result in an underestimation of the burden of disease, due to the fact that it becomes less likely that a positive sample will be read correctly. To lessen this risk, samples were quickly returned to the laboratory after being collected, and slides were read within a few hours of collection.

Similarly, the effectiveness of the drug could have played a role in the response to treatment. However, every precaution was taken in ensuring that a viable drug was obtained, and that each child took the medication correctly. Doses of albendazole were obtained from the Kintampo Municipal Hospital, and trained assistants administered the drug to each

infected child. Each child was observed taking the medication, so as to eliminate the possibility that the infected child didn't take the drug.

Other limitations of the study include the use of the questionnaire, which may not have assessed health risk factors for infection. Data was not collected on several potential risk factors including rash history, health care access in the last year, or malaria co-infection. These factors have been found to play a role in infection status and were not assessed over the course of this study. Recall bias may have played a role in response to several questions, which could have played a role in the identification of risk factors for infection. Missing data or inaccuracies in reporting were especially problematic when acquiring birth dates for each child to confirm their age. The age of each child could not always be confirmed, and data relied on the parent reporting the correct age of their child.

Conclusions

Ultimately, these data demonstrate the need for more targeted approaches to the treatment of helminth infections, as variable responses are observed within each community. Findings from Jato suggest that in some communities, chemotherapy alone is not enough. In contrast, data from other communities demonstrate that within the right setting, therapeutic approaches can make significant contributions to decreasing worm burden, at least in the short term. Yearly or bi-yearly treatment of infected children also increases the chance that a change in parasite susceptibility will occur. This is especially problematic in communities like Jato, where a lack of sanitary practices, access to latrines, and access to health education coincide with high rates of reinfection. As such, those that are cleared of infection with MDA are likely to become reinfected – thereby negating the purpose of the treatment. Given these differences in treatment response, these data suggest the need for more targeted interventions in order to further control helminth infections in the developing world. Control measures such

as health education or providing access to latrines could make a more substantial impact in communities like Jato, where MDA has been implemented, and where infection rates continue to be high among school children. Data from this study further supports the need for new approaches to combat the disease burden posed by helminth infections in much of the developing world.

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VI. Appendices

a. **Appendix 1**: Ghana Questionnaire, Summer 2013

IDENTIFICATION	
CHILD NAME _____ DATE OF BIRTH _____(DDMMMYYYY)_____ AGE _____ SEX □ MALE 1 □ FEMALE 2 HEIGHT _____(XX.XX cm)_____ WEIGHT _____(XX.XX KG)_____ SCHOOL/CLASS_____ CHILD ID #_____ HEAD OF HOUSEHOLD _____ RESPONDENT NAME_____ RELATIONSHIP OF RESPONDENT TO CHILD_____ HOUSE NUMBER……...…….. COMMUNITY…….……..... GPS LATITUDE AND LONGITUDE…… INTERVIEWER NAME _____ NUMBER _____ INTERVIEWER NAME _____ NUMBER _____ QUESTIONNAIRE ANSWERS REVIEWED _____ (DDMMYYYY Date) _____ (Initials)	

1. SOCIOECONOMIC INDICATORS			
NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	ENTER #
1.1	What is the main material of the floor?	NATURAL FLOOR.....1 CEMENT FLOOR.....2 TILE FLOOR.....3	
1.2	What is the main material of the walls?	Mudbrick .....1 Brick .....2 Other 66	
1.3	What is the main material of the roof?	Thatch .....1 Metal .....2 Other 66	
1.4	What type of fuel does the household mainly use for cooking?	ELECTRICITY.....1 NATURAL GAS.....2 BIOGAS.....3 KEROSENE.....4 CHARCOAL .....5 FIREWOOD/STRAW .....6 DUNG.....7 OTHER_____66 (SPECIFY)	
1.5	Does your household have: 1.5a 1.5b 1.5c 1.5d 1.5e 1.5f	YES NO ELECTRICITY.....1 2 RADIO.....1 2 TELEVISION.....1 2 TELEPHONE.....1 2 REFRIGERATOR.....1 2 DVD/VCR 1 2	
1.6	Does any member of the household own:	YES NO BICYCLE.....1 2 MOTORCYCLE/SCOOTER.....1 2 CAR/TRUCK.....1 2	
1.7	Does any member of the household own agricultural land?	YES.....1 NO.....2 DON'T KNOW.....88	
1.8	Does any member of the household own at least one: 1.8a 1.8b 1.8c 1.8d 1.8e 1.8f 1.8g 1.8h	How many? COW.....- HORSE.....- DONKEY .....- GOAT.....- SHEEP.....- POULTRY.....- DOG.....- PIG.....-	

1.9	Does anyone in the household own a savings account?	YES NO DK BANK……..1 2 88 CO-OPERATIVE.....….1 2 88	
1.10	How far is the household from the nearest health facility?	LESS THAN 1KM……..…1 BETWEEN 1 AND 5KM..……..….2 BETWEEN 5 AND 10KM…….....3 GREATER THAN 10KM..…….....4 DON'T KNOW……88	
1.11	How many people in the household? 1.11a 1.11b 1.11c 1.11d 1.11e	Total number _____ ≤ 5 yrs _____ 6-11 yrs _____ 12-15 yrs _____ Women > 15 _____ Men > 15 _____	
1.12	What is the primary religion of the household?	Muslim Christian Traditional Other	
1.13	What tribe do you belong to?		

2. HOUSEHOLD WATER SOURCES			
NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	ENTER #
2.1	What is the main source of water for members of your household?	PIPED WATER…….…...10 DUG WELL....……..…..…. 11 WATER FROM A SPRING…..... 12 RAINWATER…..…… 13 SURFACE WATER…..…… 14 OTHER…… 66 (SPECIFY)	

3. TOILET FACILITIES AND GARBAGE			
NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	ENTER #
3.1	What kind of toilet facility do members of the household use?	YES NO	
	3.1a	FLUSH OR POUR..……..…1 2	
	3.1b	PIT LATRINE..…….....1 2	
	3.1c	COMPOST...……..……..1 2	
	3.1d	BUCKET…….…....1 2	
	3.1e	BUSH OR FIELD……..……1 2	
	3.1f	RIVER……..1 2	
	3.1g	OTHER_____ 66 (SPECIFY)	

4. EXPOSURE/DISEASE PREVENTION			
NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	ENTER #
4.1	Has any member of your household had deworming medication in the past year?	YES…….……1 NO..……..2 DON'T KNOW……..…....88	
4.2	Does the child own shoes?	YES…….……1 NO..…….…2 DON'T KNOW……...……....88	
4.3	If yes, does the child wear shoes daily?	Almost all the time every day……1 Some of the time every day……..……2 Some of the time, not every day……3 Rarely…….4 DON'T KNOW……...……..……....88	

5. PARASITE TREATMENT			
LINE NO.	ANTI-PARASITIC HISTORY	ANTIPARASITIC TREATMENT	TREATMENT SOURCE
	Antiparasitic treatment in the past year?	See treatment codes below	See treatment source codes below
(1)	(2)	(3)	(4)
Index Child	YES NO 1 2		
Head of Household	1 2		
Mother or Caregiver	1 2		
03 FROM TABLE 6	1 2		
04 FROM TABLE 6	1 2		
05 FROM TABLE 6	1 2		
06 FROM TABLE 6	1 2		
07 FROM TABLE 6	1 2		

TREATMENT CODES

ALBENDAZOLE……1 MEBENDAZOLE……2
PYRANTEL……3 OTHER ANTI-PARASITIC_____4
DON'T KNOW.....88 (SPECIFY)

TREATMENT SOURCE CODES

HOSPITAL…….....1 LOCAL CLINIC…..... 2
PHARMACIST…..3 LOCAL HEALER…..4
FAMILY MEMBER…..5 DRUG STORE…..…..6
OTHER_____ 66 DON'T KNOW……88
(SPECIFY)

THANK YOU for all of your help. We are very grateful for your time!

b. **Appendix 2:** Egg Extraction Protocol

Purpose: Extract eggs from fecal matter; bring eggs to a known concentration for EHA and/or concentrate into 200µl to freeze for gDNA extraction.

Items needed PER SAMPLE:

1. Feces
2. 100 ml 0.9% NaCl
3. 40 ml 0.015% Brij-35
4. 40 ml 2.18M NaNO3
5. 3 x 250 ml centrifuge bottles
6. 2 x 50 ml FALCON centrifuge tubes
7. 1 sheet 4"x4" gauze
8. 1 funnel
9. 40 ml of H2O
10. 1x 80 µm filter
11. 1x 20 µm filter
12. Filter apparatus unit
13. 200 ml H2O in a flask or beaker

PROCEDURE

1. Add ~100 ml of 0.9% NaCl directly to sample in stool collection cup

OR

2. Put needed grams of feces into 250 ml centrifuge bottle and add ~100ml 0.9% NaCl
3. Shake vigorously
4. Filter over a single layer of gauze placed in a funnel to remove large particulate
5. COLLECT FILTRATE IN CLEAN 250 ML bottle
6. Squeeze gauze to get as much liquefied sample as possible
7. Centrifuge @500g for 5 minutes
8. Carefully decant supernatant-DISCARD into beaker-EGGS IN PELLET
9. Resuspend pellet in 0.015% Brij-35 measured to 40 ml mark on conical
10. Shake vigorously to resuspend pellet
11. Spin @500g for 5 minutes
12. CAREFULLY open bottle and discard supernatant into beaker-EGGS IN PELLET
13. Add 40 ml with NaNO3 (2.18 M, 1.185 specific gravity); GENTLY resuspend pellet by inverting
14. Transfer to a 50 ml FALCON centrifuge tube
15. Spin @500g for 10 minutes

    -WHILE SPINNING SET UP FILTRATION APPARATUS WITH 80 µm FILTER

16. Decant top 10 ml into a beaker or flask containing 200 ml water – swirl mix

17. Pour solution over 80 µm filter in filtration apparatus– apply gentle suction if necessary
    -EGGS WILL FLOW THROUGH THE 80 µm FILTER
18. Remove used filter from apparatus; Position clean 20 µm filter
19. Pour flow-through over 20 µm filter- EGGS WILL BE TRAPPED ON 20 µm FILTER
20. FOLD the 20 µm filter into a 50ml conical containing 40 ml of water; Shake to release eggs trapped on filter>> remove filter paper.
21. Spin @ 500g for 5 minutes
22. Let stand for 90 minutes
23. Gently aspirate down to approximately 1 ml >> **EGGS IN BOTTOM OF SOLUTION**
24. Resuspend gently and count 20 ul of suspension- adjust to 50 eggs in 100 µl for EHA
25. Dispense 10 wells of 50 eggs/100 µl each for EHA
26. High speed (1000g?) spin remaining eggs to pellet; remove water leaving 200 µl
27. Freeze -20°C.

SOLUTIONS: ALL SOLUTIONS REQUIRE HIGH QUALITY OR DISTILLED WATER

0.9% NaCl: per 1 liter
9 grams of NaCl per 1000 ml H2O

0.015% Brij-35: per liter
0.5 ml of 30% Brij-35 per 1000 ml H2O

NaNO3 (2.18 M, 1.185 specific gravity): per liter
185 grams per 1000 ml H2O

NEEDED FOR 150 SAMPLES:

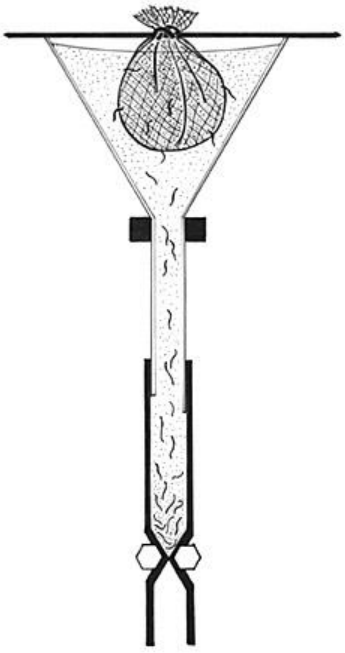
6 liters 0.9% NaCl; 54 grams NaCl
6 liters 0.015% Brij-35; 3 ml 30% Brij-35
2.25 liters 2.18M NaNO3; 416.25 grams NaNO3
150-300 50 ml FALCON centrifuge tubes
300 x 15 ml FALCON centrifuge tubes
150 sheets gauze
1 funnel
20 ml of H2O
150 x 80 µm filter
150 x 20 µm filter

c. **Appendix 3:** Baerman Funnel Set-up

1. Mix feces with bone charcoal (http://www.ebonex.com/) so that there is an even distribution. We have found a 5:1 (charcoal:feces) ratio is optimal. If soil is used it should be sterilized. The mixture is moistened with room temperature distilled water is applied so that the mixture is wetted; **water droplets should not be visible** and placed into a 150 x 20mm falcon sterile petri dish (falcon #1013). The prepared dish is incubated at 26°C in the dark for 12-14 days. A beaker of water is kept in the incubator to humidify (if necessary).

2. The plate is removed from the incubator after 12-14 days. The mixture is placed within a large kimwipe that has been folded in half over a mesh screen/sieve to create a pouch. Care is taken to contain the mixture within the kimwipe to avoid loss of sample and potential charcoal contamination of later steps. The kimwipe serves as a filter for the migrating larvae emerging from the mixture.

3. Water is preheated to 37-40°C. A funnel is set up so that it hangs vertically. Tubing has been secured to the bottom of the funnel and a clamp is in place. The kimwipe/screen/sample is placed inside a funnel such that the diameter of the screen fits within the middle diameter of the funnel. The preheated water is poured slowly over the top of the sample until the surface is submerged by 1-2 cm of water and the tubing below is full. Again, care is taken to keep the charcoal mixture within the kimwipe as the larvae do not survive well in dirty water. The air bubble that develops in the tubing over the top of the clamp is "burped" to remove.



4. The funnel apparatus is left at room temperature (22°C) for approx. 18 hours. For funnels set up with ~1000ml water--the bottom 2 X 50 mls of water in the tubing is decanted into 2 sterile 50 ml conicals by opening the clamp slightly. The sample is left undisturbed at room temperature on the bench for at least an hour to allow the emerged larvae to settle to the bottom. The top 45ml of liquid is then carefully removed and clean water is added to the remaining 5ml. This washing step is repeated 2 times to remove any contaminated water.

5. The final remaining 5 ml containing the larvae are visually assessed to confirm the presence of larvae. The volume is increased with **1X BU buffer** (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 70 mMNaCl; pH 6.8) for storage (10L3/µl).

10X BU Buffer= 500 mM Na_2HPO_4, 220 mM KH_2PO_4, 700 mMNaCl; pH 6.8; filter sterilize

d. **Appendix 4:** EGG HATCH ASSAY (EHA)

Purpose: Calculate the percent hatching of individual samples in a panel of albendazole concentrations (0, 0.1, 1, 2, and 5 µg/ml) to screen for resistant phenotypes.

Supplies:
6 - 50ml conicals for Albendazole solutions

Procedure:
- Prepare a 5 mg/ml solution of albendazole resuspended in methanol (MeOH)
- Prepare by dissolving 0.2 grams in 40 ml MeOH
  NOTE albendazole will form a milky solution- NOT CLEAR
- Prepare a 20 µg/ml solution by adding 200 µl of 5 mg/ml solution to 50 ml H2O

- Then:

Prepare the following solutions:
0 µg/ml…….Add 200 µl of methanol to 50 ml of water
2 µg/ml…….……Add 5 ml of 20 µg/ml solution to 45 ml water
4 µg/ml…….…Add 10 ml of 20 µg/ml solution to 40 ml water
10 µg/ml……..Add 25 ml of 20 µg/ml solution to 25 ml water
20 µg/ml……..Use 20 µg/ml solution

- For each sample to be tested add 100 µl per well of the above solutions in duplicate to wells of a 96 well plate.
  PLATE SET UP: ***NOTE*** **Final concentrations to be tested reflect the dilution of adding 100 µl of extracted hookworm eggs to the above solutions**
- Add 100 µl of appropriate stock ABZ solution to each well.
- Add 100 µl (50 eggs) of the egg sample to be tested to each well.

*****Final volume per well= 200 µl*****

Subject 1	0 µg/ml Repl 1	0 µg/ml Repl 2	1 µg/ml Repl 1	1 µg/ml Repl 2	2 µg/ml Repl 1	2 µg/ml Repl 2	5 µg/ml Repl 1	5 µg/ml Repl 2	10 µg/ml Repl 1	10 µg/ml Repl 2
Subject 2	0 µg/ml Repl 1	0 µg/ml Repl 2	1 µg/ml Repl 1	1 µg/ml Repl 2	2 µg/ml Repl 1	2 µg/ml Repl 2	5 µg/ml Repl 1	5 µg/ml Repl 2	10 µg/ml Repl 1	10 µg/ml Repl 2

******CONFIRM THE ADDITION OF EGGS TO EACH WELL USING A MICROSCOPE******

- Incubate the sample plate at 27°C for 48 hours.
- Record the number of larvae and the number of unhatched eggs for each treatment.
- Calculate % hatching as follows:

$$\frac{\text{Number of larvae}}{(\text{Number of unhatched eggs} + \text{Number of larvae})} \times 100 = \%\ \text{hatching}$$