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Molecular techniques to isolate soil-transmitted helminth β-tubulin genes to enhance global surveillance of benzimidazole resistance

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2016

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An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Epidemiology
2018

Abstract

Molecular techniques to isolate soil-transmitted helminth β-tubulin genes to enhance global surveillance of benzimidazole resistance

By Amanda Kobokovich

Background: Soil-transmitted helminths (STH) are highly endemic among resource-poor populations and result in serious health consequences, particularly among pre-school and school-aged children. Mass drug administration (MDA) with benzimidazoles is a highly utilized method to treat populations in these endemic areas. There is increasing concern of anthelmintic resistance to benzimidazoles, heralded by the devastating consequences of overuse in the veterinary field. There is evidence that single-nucleotide polymorphisms (SNPs) in the β-tubulin genes of the STH can confer resistance to benzimidazoles. We assessed the prevalence of these SNPs in two different study populations with different exposure to preventative chemotherapy campaigns.

Methods: Samples selected from surveillance efforts in the Solomon Islands and Guatemala to optimize PCR and Next Generation sequencing to isolate the β-tubulin genes of *Ascaris lumbricoides* and *Necator americanus* in order to evaluate the prevalence of SNPs conferring benzimidazole resistance. Statistical analysis was performed on all three STH of interest to evaluate the associations between infection and demographic characteristics.

Findings: Despite the use of a highly specific and valid sequencing technique, we found no evidence of benzimidazole resistance via β-tubulin SNPs in these populations. Regardless of lifestyle similarities, Kwai village had lower odds of infection compared to Ngongosila village across all three STH (*A. lumbricoides:* OR=0.50, 95% CI=0.36, 0.68) (Hookworms*:* OR=0.59, 95% CI=0.40, 0.86) (*T. trichiura:* OR=0.83, 95% CI=0.02, 0.37). Age was associated with both STH prevalence and low STH infection intensity for *A. lumbricoides* and hookworms. In Guatemala, odds of infection with *A. lumbricoides* was lower among hospital patients (OR=0.28, 95% CI=0.11, 0.73), while odds of infection with *T. trichiura* was higher among hospital patients (OR=4.20, 95% CI=1.81, 9.73), both compared to health center patients.

Conclusions: This is the first study of its kind to use this novel molecular technique to identify benzimidazole resistance in STH. Our analysis of infection prevalence and demographic data revealed several interesting associations between age and village, in the Solomon Islands, and site of admission, in Guatemala. More attention must be given to stopping the chain of transmission in endemic locations such that preventative chemotherapy with benzimidazoles is no longer so heavily relied upon.

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ACKNOWLEDGEMENTS

Special thanks to the members of the CDC Parasite Diagnostics and Biology laboratory, especially Fernanda Nascimento and Dr. Joel Barratt for all their assistance and technical support in acquiring genetic results. Thanks to Dr. Matthew Freeman and Dr. Richard Bradbury for all of their input and advice.

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BACKGROUND/LITERATURE REVIEW

Soil-transmitted helminth disease and current control efforts

Soil-transmitted helminths are intestinal nematodes belonging to a categorized as neglected tropical diseases (NTDs) by the World Health Organization (WHO) that disproportionally affect the world's poorest populations (1; 2). According to the WHO, soil-transmitted helminths (STH) are endemic in 149 countries, resulting in high levels of associated morbidity among the millions of at risk persons (2). Collectively, approximately 2 billion people are infected with at least one STH associated with greater than 6 million disability-adjusted life-years (DALYs) (3). Correlated to the "neglected" status of STH, many of these infected people live in impoverished communities that lack adequate water, sanitation, and hygiene (WASH) facilities (3). Morbidity from STH is particularly high among children under 14 years of age (4), where these infections lead most commonly to morbidities such as delayed growth and development, iron deficiency anemia (4) and potentially cause impediment to learning (5; 6; 7). Pregnant women are at higher risk of morbidity related issues because of poor birth outcomes associated with hookworm infection, particularly from anemia resulting from infection (8). Due to their long-lasting impacts on the growing workforce, STH infections hinder the socioeconomic growth of developing countries (2).

To address the effects of STH on the most impoverished of the world's people, the WHO has set several goals to control and interrupt the transmission of STH by 2020 (1; 2; 4). The primary mechanism by which WHO has proposed to achieve this is by

setting a goal that 75% of preschool-aged and school-aged children should be receiving preventative anthelmintic chemotherapy by 2020 (4).

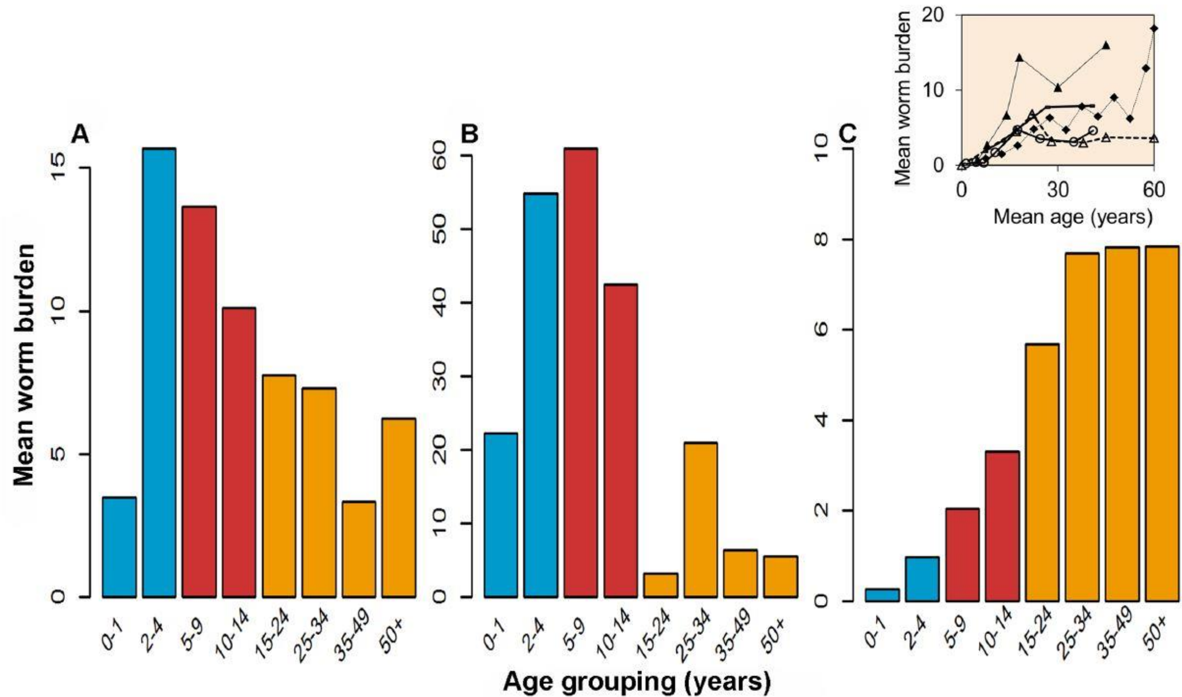

Figure 1. Taken from Anderson 2015. Cross-sectional survey data of mean infection intensity among different age classes for (A) *Ascaris lumbricoides*, (B) *Trichuris trichiura*, and (C) the hookworms (*Necator americanus* and *Ancylostoma duodenale)*. The colors represent the different age classes: pre-school age children (blue), school-age children (red), and adults (orange).

The STH are comprised of two distinct species and a third species group of human intestinal helminths, these being *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworms (3). The population prevalence of these helminths varies with age: with peaks of ascariasis and trichuriasis in preschool-aged children, while school-aged children and adolescents present with higher rates of hookworm infection (Figure 1) (3). Additionally, relatively few individuals harbor high intensity infections while most infected persons harbor low intensity infections (9). These “super-shedders” disproportionately contribute higher fecal egg counts into the environment than people

with lower worm burdens (10). Therefore, it is important to screen for anthelmintic resistance in humans, particularly for these persons with high intensity infections who may be introducing higher numbers of anthelmintic resistant worms into the environment.

Ascaris lumbricoides

A. lumbricoides is one of the most prevalent helminth infection worldwide, with 1.2 billion people infected and accounting for over 10.5 million DALYs (11). Although it is found globally, it is most highly prevalent in sub-Saharan Africa, the Americas, and Southeast Asia (11). The life cycle of *A. lumbricoides* begins with fecal-oral transmission of unembryonated eggs into the host (11). After entry into the host, the eggs move into the small intestine where the larvae hatch and migrate to the liver before finally proceeding to the lungs (11). Once the larvae enter the lungs, they move through the alveolar space and enter the pharynx where they are re-ingested and return to the small intestine to mature and mate (11). This migratory life cycle causes several clinical conditions, particularly in individuals with a high worm burden. Children tend to have the highest worm burdens because of host-characteristic preferences of *A. lumbricoides* which are still incompletely understood (12). These clinical conditions, which can be acute and chronic, include difficulty breathing, lung inflammation, abdominal pain, and diarrhea (11).

Additionally, pigs can act as a reservoir for *A. lumbricoides* and have been shown to be able to cross-infect humans (11). *A. lumbricoides* and the porcine roundworm *A. suum* can infect both pigs and humans (11). Ecological factors also influence the distribution and prevalence of *A. lumbricoides*. *A. lumbricoides* ability to survive at cooler temperatures provides with a selective advantage over *T. trichiura* and the

hookworms, a factor associated with its higher overall prevalence worldwide (13). Although the geographical range *A. lumbricoides* can inhabit is larger than the other two main STH, *A. lumbricoides* prefers areas with high humidity and relatively warm temperatures (13). However, *A. lumbricoides* does not develop well in environments with temperatures exceeding 38-40°C (13). These ecological preferences make *A. lumbricoides* particularly successful in reproducing in the tropics. Finally, *A. lumbricoides* differs from hookworms in its preferred human host age-range. Pre-school aged and school-aged children harbor both the highest prevalence and the highest intensity infections compared to adults for *A. lumbricoides* (Figure 1) (11). However, this distribution also is linked to human behaviors in the various age groups: children are easily infected via a fecal-oral route due to commonly putting unwashed hands in their mouths (14). Adults working barefoot in the fields are more likely to be exposed to hookworms which can enter through the soles of the feet (14).

Trichuris trichiura

The whipworm, *Trichuris trichiura*, is the second-most prevalent soil-transmitted helminth with approximately 795 million infected persons worldwide, 86 million of whom are under the age of 5 (7). Like *A. lumbricoides*, it is highly prevalent in tropical and sub-tropical regions (10). *T. trichiura* does not have a known zoonotic reservoir that is able to cross-contaminate humans; although, there have been few reports of the pig whipworm *T. suis* infection in humans maintaining viability for a short period of time (10). Additionally, *T. vulpis* in dogs has shown the ability to infect humans (10). *T. trichiura* also enters a human host as embryonated eggs via a fecal-oral route (10). Once they have been ingested, the embryonated eggs travels to the caecum where they hatch

and eventually reach sexual maturity (10). Similar to *A. lumbricoides*, *T. trichiura* infections are largely low intensity infections with relatively few individuals harboring high intensity infections (10).

T. trichiura, like *A. lumbricoides*, prefers climates that are warm and humid (10). Its development is optimized between 28-32°C and begins to rapidly decline in development above 38-40°C (13). However, *T. trichiura* eggs are more prone to desiccation than *A. lumbricoides*, while the embryonated eggs *A. lumbricoides* remaining viable for 5-9 days after *T. trichiura* egg development has halted at 77% relative humidity (10). Finally, *T. trichiura* is more highly prevalent among pre-school aged and school-aged children than among adults (Figure 1) (1).

Hookworms

There are three main hookworm species that infect humans: *Necator americanus*, *Anclystoma duodenale* (15) and *Ancylostoma ceylanicum* (16). Globally, over 740 million people are infected with these parasites (7). Due to similarities in their life cycles, clinical impacts, and morphologies, they are not often differentiated in the literature; however, *N. americanus* has a wider geographical range than *A. duodenale* making it the more prevalent hookworm species to infect humans (6). Additionally, *N. americanus* is also the native hookworm of Asia, the Americas, and Africa whereas *A. duodenale* has beem introduced from the Northern hemisphere (5). These hookworm species both enter the human body as third stage infective larvae through contact to exposed skin, usually the soles of the feet (6). Both species of hookworm follow a similar life cycle while in the human host as *A. lumbricoides*. Specifically, once they have entered the host, they travel the circulatory system to reach the pulmonary system

where they enter the pharynx and are swallowed (6). At this point, the hookworms can reach the intestines and mature into adults who then sexually reproduce (6). Hookworm suck blood from the host and thus infection leads to anemia in moderate to high intensity cases, which then can cause other associated problems such as poor birth outcomes in women of childbearing age (15). *A. duodenale* sucks more blood than *N. americanus* and therefore leads to worse instances of anemia in their hosts (6).

Hookworms can survive higher temperatures than either *A. lumbricoides* or *T. trichiura* (13). This could possibly be due to the fact that hookworm larvae are motile in the soil and can move downward to avoid desiccation (13). In spite of this, hookworm larvae are also more susceptible to colder temperatures than either *A. lumbricoides* or *T. trichiura* (13). Still, *N. americanus* is able to survive at lower temperatures and higher altitudes than *A. duodenale* (17). Hookworm prevalence is highest in tropical and sub-tropical areas due to prevailing heat and high humidity conditions (13). Hookworms have a much different age distribution than either *A. lumbricoides* or *T. trichiura*. They preferentially infect older school-age children and adults, while having a much lower prevalence and intensity among pre-school age children (Figure 1) (4). Still, relatively few people in the population carry high intensity hookworm burden relative to the number of persons infected (6).

Mass Drug Administration

Current drugs and drug development

The WHO currently recommends four drugs for use in mass drug administration to combat STH infections (18). These drugs are albendazole, mebendazole, levamisole, and pyrantel pamoate (19). Of these four, albendazole and mebendazole are both part of

the benzimidazole class of drugs (19). Thus, benzimidazoles are heavily relied upon for mass drug administration. Although the continuous and widespread use of these four drugs may contribute to the development of anthelmintic resistance, development of new anthelmintic drugs has been slow (3). This lack of action from pharmaceutical companies is likely a combination of the cost of creating new drugs and the impoverished status of the people who are most impacted by STH (3). Additionally, most of the anthelmintic drugs currently in use have been transitioned from the veterinary field (19).

Current mass drug administration protocol

According to the WHO, around 883 million people worldwide may require preventative chemotherapy to treat STH infections (2). To effectively control STH infections in humans, the WHO's 2020 Roadmap on NTDs set a goal for STH endemic countries to treat 75% of school-aged children with regular preventative chemotherapy (2). These guidelines do not specify goals for STH infections in the adult population. However, one meta-analysis found that the prevalence of hookworm was significantly lower after mass deworming including the adult community members compared to targeted school-level deworming (9). Another study found that increasing treatment to adult age groups over a 20-year period would be more cost-effective than continuously treating school-aged children (4). Since STH reinfection is also a key problem in transmission, treating both adults and children in the population could be more efficacious in interrupting STH transmission than just treating children alone (4). Therefore, the WHO may need to reevaluate their goals and age-range of intervention moving forward.

Efficacy of MDA for STH in humans

Some drugs are more efficacious against certain STH and less efficacious against others. The most common methods of determining the efficacy of a particular drug in field studies are the cure rate and the egg reduction rate (18; 20). The cure rate calculates the proportion of the population negative for fecal worm eggs after anthelmintic treatment (21; 22). The egg reduction rate is the percent reduction of eggs per gram of feces following treatment (21). Since benzimidazoles are heavily relied upon in MDA programs, there have been several studies conducted to determine the efficacy of benzimidazole derivatives, such as albendazole and mebendazole, against all STH of interest. Against *A. lumbricoides*, one systematic review reported high cure rates for both single-dose albendazole and mebendazole (18). However, these same drugs at a single dose were overall less efficacious against *T. trichiura* with cure rates of 28% and 36% respectively (20). Against hookworms, albendazole appears to be more effective than mebendazole as demonstrated by consistently higher cure rates across different field studies (23; 24). Still, there appears to be a general consensus that albendazole is the preferred drug of choice for both *A. lumbricoides* and hookworms; although, more research is needed to address the marked failure of benzimidazole derivatives against *T. trichiura* (18; 24).

Apart from the benzimidazoles, levimasole is also frequency used in MDA programs. One systematic review found that out of the three STH species discussed here, levimasole was most efficacious against *A. lumbricoides*, but presented disappointingly low cure rates against *T. trichiura* and hookworms (20). In fact in *T. trichiura*, levimasole cure rates did not differ significantly from placebo (20). Against hookworms,

levimasole also has poor cure rates, such as 11.9% for single-dose 40-80 mg of drug (18). However, relatively few studies have been conducted on the efficacy of levimasole against STH so it is difficult to definitely state a trend (18). One systematic review was only able to include 2 studies per STH species in their selection criteria and analysis due to the paucity of literature available on the subject (20).

Much like levimasole, there are relatively few studies examining the efficacy of pyrantel pamoate against STH compared to studies on the benzimidazoles. Against *A. lumbricoides*, pyrantel pamoate performs fairly well; one study reported an egg reduction rate of 87.9% (18). For *T. trichiura*, pyrantel pamoate produces low cure rates; one non-randomized placebo trial reported a 28.1% cure rate (18). While pyrantel cure rates were higher than levimasole cure rates against hookworms, it is still outperformed by albendazole (20; 23). Thus, single-dose pyrantel pamoate is still a good option against certain STH, though arguably the best options for treatment against all three STH of interest remains the benzimidazole derivatives.

Anthelmintic Resistance in Veterinary Medicine

Development of Resistance in Livestock

Large-scale preventative chemotherapy campaigns, or mass drug administration (MDA), have been undertaken globally to control STH infections in humans (25). However, there has been increasing concern of anthelmintic resistance developing to the relatively few drugs that are available to treat these parasites (18). This concern has arisen from the of the emergence of resistance to anthelmintic drugs in the veterinary field (18). The World Association for the Advancement of *Vet Parasitol.* (WAAVP) defines resistance as a percent reduction in egg count following drug treatment below

95% with a lower 95% confidence level below 90% as measured by a fecal egg count reduction test (FECRT) (26). Due to mass deworming practices, helminth populations in domesticated animals are under high selection pressures and have consequently developed increasing levels of resistance to anthelmintics over time (27). Pan-resistant *Haemonchus contortus* populations now infect some sheep and goat farms, for which no anthelmintics drugs are efficacious. This phenomenon is particularly associated with regions that applied mass drug administration to livestock for many years, such as the southern United States (28). In one study conducted in the US from 2002 to 2006, 22 out of 46 sheep/goat farms found that *H. contortus* populations were resistant to all three classes of anthelmintics: benzimidazoles, levamisoles, and macocyclic lactones (28). In this study, 98% of the farms studied showed full resistance to benzimidazole in their respective livestock *H. contortus* infections (28). Infections in larger domesticated animals, such as horses and cattle, to date have shown lower levels of benzimidazole resistance than sheep or goats (28). Specifically, one study found that 98% of goat farms in the United States had resistant *H. contortus* populations while another study found that onl 74% of cattle farms in New Zealand showed resistance to albendazole (28). These parasites include *Coopera* species, *Osteragia* spp., and *Haemonchus* spp. in cattle with most drug failure recorded against benzimidazole and ivermectin class drugs (28). In horses, *Parascaris equorum* resistance to ivermectin is of major concern, although benzimidazoles still appear to be effective in these populations (28). However, benzimidazole resistance in horse intestinal helminth infections has been increasing in recent years, causing significant concern in the large animal veterinary world (28).

Although anthelmintic resistance in veterinary practice appeared to emerge suddenly, resistance actually emerged following a sigmoidal curve in several species over some years (29). This pattern is characterized by relatively few cases of apparent drug failure over a long period of time are quickly followed by a sudden spike in widespread resistance, at which point resistance is irreversible (29). This pattern previously observed in livestock signals the importance of investing in more sensitive techniques to identify cases of anthelmintic resistance in humans before such an exponential explosion occurs (29).

Molecular mechanisms of benzimidazole resistance

The benzimidazole class drugs, which includes albendazole, mebendazole, fenbendazole, and oxfendazole, have been used in the veterinary field to control helminthiases since the 1960s (30; 31). Benzimidazoles attack parasitic worms by binding to crude β-tubulin subunits in the worm's cells which prevents the formation of microtubules (32). Without adequate microtubule formation, cell function is greatly decreased, and the worms subsequently die and are passed in the host's fecal excretions (32).

There is a preponderance of evidence to suggest that mutations in the β-tubulin isotype 1 and isotype 2 genes can confer resistance to benzimidazoles in STH (33; 31). The mode of action of benzimidazoles is to bind with high affinity to the β-tubulin during polymerization of microtubules (33). The mutations in β-tubulin isotype 1 and isotype 2 genes decreases the affinity of benzimidazoles for their typical binding sites on the β-tubulin dimer subunit (33). This change in affinity is accompanied by a loss of alleles at

the β-tubulin isotype 1 locus (29). These mutations occur through single nucleotide polymorphisms (SNP) at coding points which lead to specific amino acid substitutions in the resultant β-tubulin protein (31). The most common SNP to confer benzimidazole resistance is in β-tubulin isotype 1(35). This point mutation involves a thymine-to-adenine transversion at codon position 200, which results in a change from phenylalanine to tyrosine (31). The expression of this F200Y *H. contortus* alleles in *C. elegans* was sufficient to produce benzimidazole resistance (35). Another point mutation, F167Y, also appears to confer benzimidazole resistance with a phenylalanine-to-tyrosine transversion in β-tubulin isotype 1, via an adenine-to-thymine point mutation at codon 167 on the β-tubulin gene (31). A third candidate for benzimidazole resistance mutations and involves an adenine-to-cytosine transversion SNP at codon 198 in β-tubulin isotype 2 which results in a change from glutamate to alanine (35). While benzimidazole resistance appears to be highly correlated with SNPs at these three locations, more research is required to determine if there are other areas in any STH genome that may contribute to resistance (36).

Resistant-type STH differ genotypically as well as phenotypically from susceptible-type STH. Egg-hatch assays on *H. contortus* revealed that genotypically resistant-type worms had 4 to 10 times higher IC_{50} values compared to genotypically susceptible-type worms when treated with thiabendazole (35). However, there is most likely a fitness cost associated with being homozygous recessive which is supported by the low overall frequency of resistant alleles in the population before drug treatment (37). Many researchers have hypothesized that these resistance alleles have a slight deleterious fitness effect, but upon continuous selection pressure from anthelmintics, genotypic

compensations occur to re-associate better fitness characteristics with resistance characteristics (37). Thus, over time the deleterious fitness effect of having one or more resistant alleles decreases as more homozygous recessive and heterozygous worms comprise a larger proportion of the population (37).

Besides benzimidazoles, other anthelmintic drug classes have been shown to have decreased efficacy against STH in recent years. However, less is known about the exact mechanism of resistance to these drugs. For levimasole, resistance is thought to be caused by a decrease in affinity for the drug to bind to nicotinic acetylcholinesterase receptors or a decrease in the number of these receptors in the cell (29). Little is known about the mechanism for ivermectin (a macrocyclic lactone class drug) resistance, but it is hypothesized that a mutation in the alfa-subunit of chloride-gated channels decreases ivermectin's mode of action (29). Still less is known of the mechanism of antischistosomal drugs, such as praziquantel and oxamniquine (29). However, the absence of a sulfo-transferase activating enzyme in resistant-type schistosomes is a promising lead for future research (29).

The concept of refugia and its influence on the epidemiology of anthelmintic resistance

An important concept in the epidemiology and control of anthelmintic resistance is refugia. Refugia is the proportion of the population that is not treated with infection control measures and is therefore not susceptible to increased selection pressures (Figure 2) (38). Additionally, since the worms undergo sexual reproduction, non-random mating could more quickly select for resistant genes, particularly under high selection from relatively few drugs (27). Therefore, it is important to manage both the homozygous- and

heterozygous-resistant population to slow or prevent the saturation of resistant alleles among the herd (29). In this way, anthelmintic resistance is different from antibiotic resistance because resistant allele frequencies are subject to change from generation to generation due to non-random mating selection. One study demonstrated that changes in allele frequencies contributed more to the development of benzimidazole resistance in *H. contortus* than random mutations or novel rearrangements (29). By leaving some of the population untreated, the overall drug-resistant parasite population is diluted by the overwhelming presence of drug-susceptible parasites (39). This management results in lower selection pressures that would increase the drug-resistant population (39). Refugia can be used to effectively manage the development of drug resistance by balancing drug coverage and mixing allowed between treated and non-treated populations (39). In one study, the frequency of resistance genes in a population was always higher for animals in low refugia compared to animals in high refugia, regardless of treatment schedule and parasite fitness disadvantages (40). Another study suggested that by leaving 20% of the population untreated, the development of anthelmintic resistance is greatly slowed (29). Thus, refugia remains a viable option for the control of anthelmintic resistance in animals and a possibility for control in human populations.

It has been suggested that mutations in the β-tubulin gene which confer resistance to benzimidazoles also decrease the overall reproductive fitness of the organism (40). Models have shown the decreased fitness of parasites with one or more point mutations at the three alleles of interest compared to fully susceptible parasites (40). Therefore, homozygous recessive worms would theoretically have a better chance of surviving anthelmintic drug treatment, but a lowered overall reproductive fitness (40). This

dichotomy is what makes refugia management so important; if the population of resistant worms is highly diluted in the susceptible population, resistance will develop at a slower rate due to fitness costs (40).

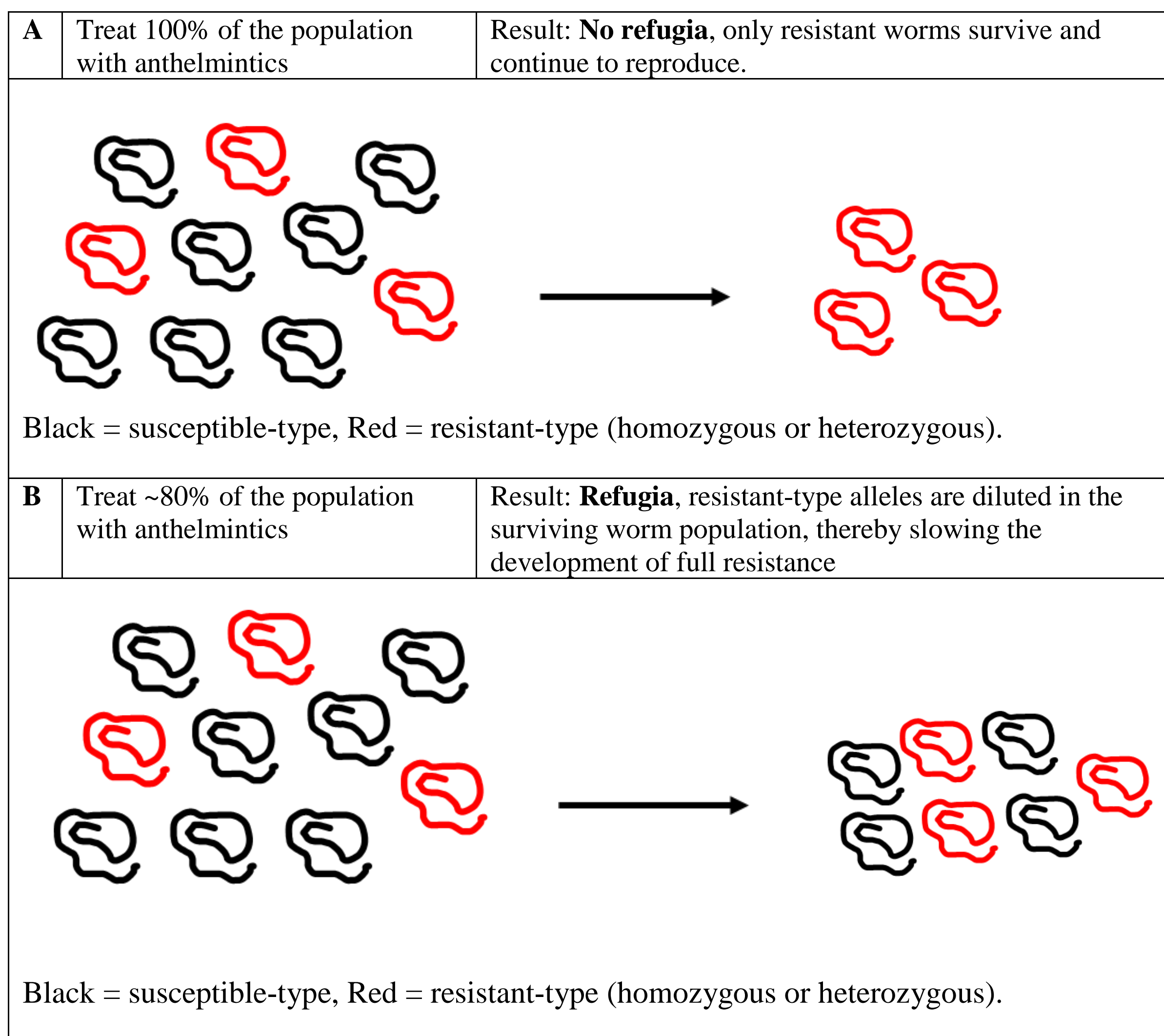

Figure 2. Describes how refugia dilutes the number of resistant parasites and resistant alleles in population receiving anthelmintic drugs. In (A), the entire population is treated with anthelmintics, resulting in the survival of only resistant worms. In (B), refugia management is demonstrated by treating only 80% of the population as suggested in Geerts et al. 2000.

The Unseen Threat of Anthelminthic Resistance in Human STH

For several years, there have been sporadic reports of the reduced efficacy of benzimidazoles in field trials, particularly against hookworms (8; 23; 41). These studies

describe the lowered efficacy of albendazole and mebendazole when given in single doses to populations with high prevalence of hookworm infections. One study in Haiti found a statistically significant increase in the homozygous resistant-type of *T. trichiura* at both codon position 200 and 198 following treatment with albendazole (42). Additionally, albendazole efficacy in this population decreased between pre-treatment and post-treatment as evidenced by fecal egg count reduction test scores of 48.4 vs. 24.5, respectively (42). This study supports the hypothesis that MDA in humans may be increasing selection pressures for anthelmintic resistance in STH. Although lower cure rates have been described for these drugs, the existence of anthelmintic resistance in human STH still remains unconfirmed via molecular techniques (8). Despite increasing evidence of anthelmintic resistance in the literature, it is difficult to compare drug efficacies between studies due to variation in dosing regimens and diagnostic tests (21). This discrepancy is particularly true for the cure rate (CR) method of quantifying drug efficacy. The cure rate measures the percent of the population negative for fecal worm eggs after drug treatment (21; 43). Unfortunately, because of its high variability in situations where infection intensity is low it is not an ideal method of measuring drug efficacy in human populations (21; 43). Therefore, there is need for standardized methods of assessing drug efficacy, both field-based and molecular (24).

Detecting of Anthelmintic Resistance in STH

Field-based assays

The strengths and limitations of two field-based assays for the detection of anthelmintic resistance are described below: the fecal egg count reduction test (FECRT) and egg hatch assay (EHA). First, the FECRT is a trusted test in the veterinary field to

monitor drug efficacy because it can be used across all anthelmintic groups (44). The standard procedure is described in a review by Coles et al. (Figure 3) (44). However, the FECRT is only reliable when at least 25% of the worms in a population are

(A) Fecal Egg Reduction Count Test in Sheep/Goats
1. Randomly distribute or distribute based on egg counts.
2. Choose animals 3–6 months of age or if older with eggs counts >150 epg.
3. Use 10 animals per group if possible.
4. Rectal sample putting 3–5 g into individual pots.
5. Count using the McMaster technique as soon as possible after collection.
6. Only store at 4 8C for 24 h if using samples for culturing.
7. Individually weigh animals and give manufacturers recommend dose orally, from a syringe.
8. Take second rectal sample at the following time periods after treatment:
   Levamisole 3–7 days.
   Benzimidazole 8–10 days.
   Macrocyclic lactones 14–17 days.
9. If testing all groups in same flock, collect at 14 days.

(B) Egg Hatch Assay
1. Add 1.89 ml water to each well in a 24 well plate. This should be deionised water with a neutral pH. Then add 10 ml of thiabendazole (Sigma-Aldrich T8904) solution dissolved and diluted in DMSO to the water. Add DMSO to the control wells. Do not dilute DMSO solutions of thiabendazole in water.
2. To determine the degree of resistance use 0.05, 0.1, 0.2, 0.3 and 0.5 mg/ml thiabendazole. A single concentration of thiabendazole can be used, the discriminating dose (see text).
3. Place 100 ml of fresh eggs (less than 3 h old or anaerobically stored) in each well. Since thiabendazole may bind to debris the eggs should be as clean as possible. Incubate at 25 8C for 48 h.
4. Add two drops of Lugol's iodine to each well. Count at least 100 of the remaining eggs and hatched larvae. Either count directly using an inverted microscope or carefully wash eggs and larvae out of the well onto a microscope slide or petri dish marked with a grid and count on a compound/ binocular microscope.

Figure 3. Modified from Coles, *et al.* 2006. Describes the procedures for the FECRT (Panel A) and the egg-hatch assay (Panel B). The FECRT test described here is specifically for sheep and goats.

resistant (45). In human populations, particularly in resource poor communities that already have limited access to healthcare, by the time the FECRT detects resistance, the consequences of high anthelmintic resistance will likely be irreversible (45). Additionally, there is also the potential to over- or underestimate true drug efficacy due to the wide range of analytic sensitivity (46).

Another field-based assay that can be used in human populations is the egg hatch assay or EHA. The standard procedure is described in a review by Coles, *et al.* (Figure 3) (44). The EHA has only been optimized from veterinary purposes for human hookworms (45). Still, it is a good option for use in resource poor settings because of its minimal costs and low labor intensity to obtain reliable results (36). However, the EHA is not as reliable in situations where there is a low intensity of infection (36). Therefore, the EHA is less broadly useful compared to the FECRT in human populations. Thus, there is a need for more sensitive assays to detect anthelmintic resistance in humans before resistance reaches irreversible levels.

Molecular techniques

It is important to develop more sensitive and specific molecular techniques to identify resistant worms before they overtake the sensitive worm population (35). Molecular techniques may be preferable over field-based assays to detect resistance since many of those tests require adult worms; genomic tests can be performed on a parasite in any life-stage (35). Because these point mutations are heritable and have been widely found in different animal populations, these are reliable molecular markers for the detection of benzimidazole resistance (27). PCR and real-time PCR assays are able to detect the presence of these SNPs in a quick and reliable manner, given sufficient knowledge of the genome sequence in the parasite of interest (47). For real-time PCR in particular, it is critical to know the melting point for each particular SNP, which are both species specific and specific to the region being amplified (47). Conventional PCR must be followed by Sanger sequencing in order to reveal whether or not a SNP has occurred at the β-tubulin alleles of interest (36). Several studies have been performed to optimize

PCR on human parasites for this purpose (36; 47). In the context of mass drug administration, where the target populations are usually in resource-poor settings, these molecular assays can be more cost effective and standardized than other detection techniques (3). However, if the mutation that confers resistance is not one of the three aforementioned SNPs, then it will not be detected through these methods (44). The most limiting factor in the currently applied molecular assays for detecting β-tubulin gene mutations associated with benzimidazole resistance is the presence of multiple mating adult worms in infected humans, which may have any individual array of anthelmintic resistance genes (48). If single or pooled fecal samples were to be tested, multiple genotypes would result, with often only the most prevalent being detected (49). When conventional PCR and sequencing is applied, chromatographs with pooled samples will be extremely difficult to interpret (31). To resolve this, current assays rely on the laborious process of isolating single eggs or larvae from fecal samples and testing these individuals by PCR (48). This approach not only lacks the sensitivity required to low level prevalence of resistant nematodes in a single host, but is wholly unsuitable for mass, population level screening (31).

The current state of anthelmintic resistance in human STH worldwide

Thus far, there has been no confirmed instance of benzimidazole resistance in human STH using molecular techniques; although, decreased efficacy to albendazole and thiabendazole have been reported (27; 42; 47). This discrepancy highlights the need for more frequent monitoring and improvement of our current knowledge of benzimidazole resistance in human STH populations. In particular, Diawara et al. describe a significant increase in homozygous- and heterozygous-resistant *T. trichiura* following a single dose

albendazole treatment to a previously albendazole-naïve Haitian community (42). When the genotypes of these worms were assessed two weeks post-treatment, the researchers observed that the homozygous resistant-type at codon position 200 had increased from 3.1% to 55.3% (42). These findings support the hypothesis that MDA could be driving changes in allelic frequencies in STH populations that could result in widespread anthelmintic resistance in humans.

There are several factors that could be driving decreased drug efficacy and possible anthelmintic resistance in humans. First, geographic location appears to play a role in drug efficacy. Lower cure rates have been observed for albendazole in Southeast Asia than in other areas of the world (21). This discrepancy could be due to genetic differences in the regional parasite populations, host diet, and the types and quality of anthelmintics used (21). Diet also appears to play a role in drug efficacy, impacting the absorption of the drug (24; 50). The treatment frequency may also speed the development of drug resistance by increasing selection pressure on the parasite populations with several rounds of treatment per year (51). Martin, et al. demonstrated that the regular thiabendazole treatment schedule for sheep (5 times per year) produced egg hatch assay LC_{50} values in *Osteragia* spp. that were 14 times higher compared to theLC_{50} values of worms from sheep that were never treated with thiabendazole (52). Even through human populations typically only experience 1-3 rounds of treatment per year, this number of treatments was enough to drive anthelmintic resistance in sheep and goats (53). Single treatment protocol is most often used in MDA context because it is easier to ensure even distribution among the target population than multi-day drug courses (41). However, single doses of benzimidazoles may not be sufficient to kill all

parasites within a person, which provides greater opportunity for resistant worms to develop and procreate. In particular, mebendazole given in single doses seems to have a low efficacy against *T. trichiura* (23; 41). When coupled with the WHO goals for 2020, treatment frequency is likely to increase as more organizations scale-up their MDA programs (27). In spite of increasing MDA in areas with endemic STH, monitoring for drug resistance or drug efficacy is not typically part of the programs (27). Therefore, it is imperative that monitoring be implemented into MDA programs in order to detect any level of anthelmintic resistance. Even a low level of detected resistance could be warning signs for serious problems with anthelmintic resistance to come as it did in the veterinary field (53).

Summary

STH infection remains a widespread problem globally and presents many difficulties for control efforts. These difficulties range from differential drug efficacy in the three groups of parasites described above to variations in human host characteristics. The WHO has proposed a control program using MDA of anthelminthic drugs in order to treat 75% of pre-school aged and school-aged children living in endemic areas for one or more of the main classes of STH. This practice parallels MDA protocols used in the veterinary field which resulted in disastrous consequences regarding anthelmintic resistance. The development of benzimidazole resistance in STH in domestic animals has been well-documented due to over-reliance on benzimidazoles. Therefore, there is reason for substantial concern that the same pattern will occur in humans under the current WHO guidelines. Lessons from the veterinary field have taught us that we need better diagnostic tools to allow for mass screening to detect resistance before it is too late

to slow or reverse it. Techniques using Sanger PCR and deep sequencing of the β-tubulin gene in all three parasite species of interest are a promising alternative to less sensitive and specific field tests that are currently in use.

INTRODUCTION

Soil-transmitted helminths (STH), are intestinal nematodes that disproportionately affect the world's poorest populations (1). Worldwide, STH infections - predominantly *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworms (*Ancylostoma* spp. and *Necator americanus*) - are associated with greater than 6 million disability-adjusted life-years (DALYs), many of which are associated with childhood morbidity (3). While mortality from these infections are generally low, morbidity from STH can be highly detrimental to child growth and development, causing intestinal problems, such as malabsorption and diarrhea, and anemia (4). To decrease STH-associated morbidity, the WHO has set targets to control and interrupt the transmission of STH by 2020 (1; 2; 4), one of which is to provide preventative chemotherapy to 75% of preschool-aged and school-aged children (2). One class of anthelmintics, the benzimidazoles, including albendazole and mebendazole, are heavily relied upon, by being mandated by the WHO, for preventative chemotherapy because of their ease of dosing, low cost, and broad range of anthelmintic activity (21).

This scale up of population-level preventive chemotherapy (PC) may result in the development of anthelmintic drug resistance through repeated, frequent doses of single drugs in endemic populations. This concern is well founded due to the rapid and widespread of STH resistance to benzimidazoles in the veterinary field (18). In one study, 98% of the domestic sheep/goat farms in the United States showed full resistance to benzimidazole derivatives (28). Anthelmintic resistance in the veterinary field first appeared as relatively few cases of drug failure over a long period of time and quickly followed by a sudden spike in widespread resistance (29). In humans, there have been

sporadic reports of the reduced efficacy of benzimidazoles in global field trials, particularly against hookworms (8; 23; 41; 42). There is concern that resistance to benzimidazoles in human STH populations could be at the beginning part of the sigmoidal pattern previously observed in domestic animals. One control strategy suggested in the veterinary field is refugia management (38). By refraining from treating the entire population and only treating the animals with the highest morbidity from STH, refugia dilutes the proportion of resistant worms within the larger susceptible worm population (39). In addition to properly managing resistant worms in both human and animal populations, it is important to be able to identify those resistant worms in order to better direct future medical protocols. Many studies have suggested that mutations in the β-tubulin isotype 1 and isotype 2 genes can confer resistance to benzimidazoles in STH (31; 33). There is evidence that point mutations in these genes decrease the affinity of benzimidazoles for their typical binding sites on the β-tubulin dimer subunit (33). SNPs associated with benzimidazole resistance in both β-tubulin isotypes have been identified in three specific codons conserved across species: codons 167, 198, and 200 (34).

We performed nested PCR to amplify the β-tubulin codons of interest on fecal samples from the Solomon Islands and Guatemala to test for drug resistance. We use nested conventional PCR and Next Generation Sequencing to evaluate the prevalence of resistance-conferring β-tubulin gene SNPs using species-specific primers for *A. lumbricoides* and *N. americanus.* Few studies have performed comprehensive molecular tests on all four of the aforementioned STH species at all three codons of interest. One study found a statistically significant increase in heterozygous-resistant SNP frequency, and probably presence of homozygous-resistant SNPs via Hardy-Weinberg equilibrium

calculation, at codon 200 in *T. trichiura* populations after treatment with albendazole, but we still lack data on resistant SNPs for *A. lumbricoides* and *N. americanus* from field samples in humans (42). We also explore how population dynamics of STH infection vary between the Solomon Islands and Central America. We hypothesize that current MDA protocols may be driving benzimidazole resistance in STH. Any evidence of benzimidazole resistance should be taken as a warning signal for potential widespread failure of mass drug administration and acted upon accordingly. From these tests we assessed whether there should be an increased cause for concern of anthelmintic resistance to benzimidazoles in one or more STH populations in humans.

MATERIALS AND METHODS

Study design and study areas

Solomon Islands

This cross-sectional study was conducted by Atoifi Adventist Hospital, Atoifi, Malaita, Solomon Islands, on the two adjacent coral atolls of Kwai and Ngongosila in the Solomon Islands in August 2014. All residents of both atolls were invited to participate as part of the STH surveillance efforts with the goal of decreasing STH infections in these atolls. Each person/family unit was visited by a member of the research team in their homes and asked if they were willing to participate. Fecal samples were collected using coded containers given to each participant upon entry into the study. This code was used in place of identifying information; thus, all samples were de-identified and non-re-identifiable. At the time of coding, various demographic features of each participant were also collected such as age, sex, and religion. Participants were given pre-identification-coded sample containers and instructed to provide a fecal sample at their leisure and then drop off their samples at a collection point at the public toilets in each village. These containers were collected twice a day by the research team. Both populations were naïve to any preventative chemotherapy efforts; however, sporadic individual deworming may have occurred at the nearest health clinic which was over seven miles and one hour away by boat.

Guatemala Study

Participants for this cross-sectional study were enrolled from local health centers and hospitals as part of ongoing surveillance efforts by a collaboration between the Centers for Disease Control and Prevention, the Guatemala Ministry of Public Health and Social Assistance, and the Center for Health Studies from La Universidad del Valle in

Guatemala between 2007 to 2016. As such, some of the stool samples have been in cryo-storage for much longer than others. The study populations were from the towns of Santa Rosa, just north of Guatemala City, and Quetzaltenango in the southwest near the Mexican border. The participants were sampled from all patients arriving at the health facility sites who fit the inclusion criteria of having active diarrhea, among other criteria such as flu-like symptoms which were not used in the analysis of this study. Participants ages ranged from three months to 77 years old. Fecal samples were obtained from all participants the same day that consent was obtained. At the time of collection, demographic variables such as age, gender, and family connections were recorded. For MDA history, treatment with benzimidazoles was difficult to ascertain for individuals due to a lack of access to prior medical records. However, we know that twice-yearly ivermectin MDA has taken place in these populations since 1991 as part of the onchocerciasis elimination program so some incidental STH deworming has also taken place.

Isolation and Analysis of Species-Specific β-tubulin gene and SNPs

Extraction of STH DNA from fecal samples

Solomon Islands fecal samples had been previously microscopy confirmed and extracted using the Qiagen DNeasy PowerSoil kit and were therefore ready to proceed directly to PCR methods. All 191 Guatemala samples were selected from a larger sample from the VICO study by only including those samples which had at least one STH infection identified via microscopy. DNA extraction from Guatemalan samples was entirely performed by Amanda Kobokovich. The Qiagen DNeasy PowerSoil kit was used to extract parasite DNA from previously frozen Guatemalan fecal samples (Qiagen,

Hilden, Germany). MoBio powerbeads were replaced with Zirconia-silica beads (Sigma Aldrich) in the same screw cap tubes provided in the kit to the 0.5 ml mark. Then, 200 µl of fecal sample was added to the screw cap tubes. Following 10 minutes in a 65°C heating block, the samples that contained any STH aside from *T. trichiura* were ground for three minutes. For samples that had microscopy-confirmed *T. trichiura*, a second extraction was performed and egg grinding time was increased from three minutes to seven minutes in order to effectively extract the DNA from the tougher exterior of these eggs. From there, the extraction proceeded according to the PowerSoil kit instructions (Appendix A). STH genomic DNA was eluted twice in 50 µl nuclease-free water; one elution was used for confirmatory PCR and further testing while the other was stored at -80°C for future use.

Conventional PCR and gel electrophoresis of STH β-tubulin genes

Following confirmation of which STH species were present in each sample via microscopy, conventional PCR was performed to amplify the β-tubulin codons of interest for each applicable parasite (Table 2). PCRs were performed by both the author and staff of the Centers for Disease Control and Prevention Division of Parasitic Diseases and Malaria. In order to improve the sensitivity of the reaction for the β-tubulin codons of interest, a nested PCR was performed. PCR Primers (table 2) were designed to amplify a large species-specific β-tubulin product. Following, this nested primers were employed to amplify the internal fragment of interest from the initial PCR product. All primers were designed by Dr Fernanda Nascimento, Centers for Disease Control and Prevention Division of Parasitic Diseases and Malaria.

For *A. lumbricoides* external PCR, 1 µl of DNA was used as the template in a 25 µl reaction. Included in each reaction were 9.5 µl of UV-sterilized distilled water, 1 µl of forward primer, 1 µl of reverse primer, and 12.5 µl of Q5 HotStart High Fidelity DNA Polymerase. The temperature program for *A. lumbricoides* (external) was as follows: 98°C for 30 sec, followed by 20 cycles of 98°C for 10 sec, 66°C for 10 sec, 72°C for 15 sec, followed by 72°C for 2 min. The internal reaction then followed with 5 µl of external PCR product, 1.5 µl of forward primer, 1.5 µl of reverse primer, 4.5 µl of UV water, and 12.5 µl of Q5 HotStart High Fidelity DNA Polymerase. All reactions were placed in a conventional thermocycler. The temperature program for *A. lumbricoides* (internal) was as follows: 98°C for 30 sec, followed by 40 cycles of 98°C for 10 sec, 64°C for 10 sec, 72°C for 10 sec, followed by 72°C for 2 min.

For *N. americanus*, the first fragment containing codon 167 underwent a 20 µl reaction with the following mix: 1 µl DNA, 1 µl of forward primer, 1 µl of reverse primer, 7 µl of UV water, and 10 µl of Q5 HotStart High Fidelity DNA Polymerase. The external temperature cycle was as follows: 98°C for 2 min, followed by 40 cycles of 98°C for 15 sec and 65°C for 1 min, followed by 65°C for 5 min. The internal reaction then underwent a 25 µl reaction with the following mix: 1 µl external PCR product, 1 µl of forward primer, 1 µl of reverse primer, 9.5 µl of UV water, and 12.5 µl of Q5 HotStart High Fidelity DNA Polymerase. The internal temperature cycle was as follows: 98°C for 2 min, followed by 20 cycles of 98°C for 15 sec, 64°C for 15 sec, 65°C for 15 sec, followed by 65°C for 5 min. Finally, the second *N. americanus* fragment, containing codons 198 and 200, underwent the same external mix ratio and conditons as fragment 1. The internal fragment 2 reaction mix was as follows: 1 µl external PCR product, 1 µl of

forward primer, 1 µl of reverse primer, 7 µl of UV water, and 10 µl of Q5 HotStart High Fidelity DNA Polymerase. The internal temperature cycle was: 98°C for 2 min, followed by 20 cycles of 98°C for 15 sec, 67°C for 15 sec, 65°C for 15 sec, followed by 65°C for 5 min.

Genetic Analysis Sequencing of STH β-tubulin genes

All PCR products were cleaned using Zymo Research DNA Clean and Concentrator Kit according to the manufacturer's instructions. Sequencing was performed using the ABI 3130xl 16 capillary electrophoresis system. All PCR products were placed into the sequencing plate with 2.0 µl DNA template being combined with 13.5 µl ddH_2O, 3.5 µl 5x sequencing buffer, 1 µl primer (3.2 µM/µl), and 1 µl Sequencing Ready Reaction mix. The PCR program in the ABI machine was performed as follows: 96°C for 1 min, 30 cycles of 96°C for 10 sec, 55°C for 4 min, and a 4°C hold. All PCR products were purified using the NEB Monarch DNA Purification kit (New England Biolabs, Ipswich, MA). Illumina MiSeq next generation sequencing and bioinformatic analysis was performed by staff of the Centers for Disease Control and Prevention Division of Parasitic diseases and Malaria.

Data analysis

All statistical analysis was performed using SAS Version 9.4. All statistical analysis of demographic data was performed entirely by Amanda Kobokovich. For statistical analysis of the Solomon Islands dataset, exclusion from analysis was based upon lack of information for age, either through error at the time of collection or at the time of data entry. The dataset used for the Guatemala analysis only included 191 persons who were positive for at least one STH infection from the larger total study

population. This subset was chosen in order to aid in selecting samples to be used in genomic analysis. Chi-square tests were used to compare the association between genotype frequencies of SNPs at codon positions 167, 198, and 200 to various geographic and demographic characteristics of the study populations. Logistic regression analysis was performed to evaluate the odds of infection by sex and village, respectively. Additionally, logistic regression analysis was performed to evaluate the odds of infection as age increases, using 10-year age-groups. Logistic regression analysis was performed to evaluate the association between infection intensity and age. Low, medium, and high infection intensity, measured in eggs per gram, were independently defined for each different STH following the WHO protocol recommendations (54). Bioinformatics analysis of sequences was performed by staff of the Centers for Disease Control and Prevention Division of Parasitic diseases and Malaria in Geneious version 10.2.2 (Biomatters Limited, Auckland, New Zealand).

Ethical approval

For both studies, informed consent was obtained for all participants. Ethical approval for the Solomon Islands study was obtained from James Cook University Human Research Ethics Committee, the AAH Research Ethics Committee, and reciprocal ethics approval by the Central Queensland University Human Research Ethics Committee. All participants with STH found in their fecal samples were provided anthelmintic treatment. Ethical approval for the Guatemala study was obtained by the Internal Review Board of the Centers for Disease Control and Prevention in Atlanta, Georgia, U.S. as well as the Ethics Committee from the Ministry of Health and Social Assistance in Guatemala City, Guatemala. Participants that were not able to read the

consent form as it was written were provided an interpreter to obtain consent. Adults that were not capable of giving independent consent were enrolled based on their own agreement and the informed consent of their child/parent/guardian. For both studies, a parent or legal guardian gave informed consent for every child participant under the age of 17. Participants in the Guatemala study independently consented to have their samples stored for future studies. All samples from both locations were previously fully de-identified and non-re-identifiable. Approval for use of these samples for this thesis was given in the form of Exemption Waivers by both the Emory IRB and CDC IRB committees.

RESULTS

Study subject characteristics

Solomon Islands study

From a dataset of 999 individuals, 678 persons were included in statistical analysis. The ages of subjects ranged from less than 1 year old to 92 years old (Table 1). The mean age was 23.5 years old (SD=19.1) and 32.4 % of participants were less than 10 years old (Table 1). Half (49.9%) of participants were female. Although three villages were sampled, Ngongosila accounted for 40.3% of participants, Kwai accounted for 57.1%, and Na'au only accounted for 2.5% of participants (Table 1). The most common infection at all sites sampled was *Ascaris lumbricoides*, followed by hookworms. The predominant hookworm in the area is *N. americanus*. *T. trichiura* is relatively uncommon in this area (Table 1).

Guatemala study

The ages of subjects ranged from 3 months to 77 years (Table 1). Similar to the Solomon Islands study, this study population was fairly evenly divided between males and females with 53.3% of participants being female (Table 1). Participants were admitted to the study via one of three routes: health center, health post/outpost, or hospital. The majority of participants were admitted to the study through health facilities (49.7%) while the fewest were admitted through hospitals (19.4%) (Table 1).

Frequencies of β-tubulin SNPs in *A. lumbricoides*

We found no evidence of benzimidazole resistant SNPs through Next Generation sequencing in *A. lumbricoides* isolates from Guatemala or the Solomon Islands. 138 Samples from Guatemala and 73 samples from the Solomon Islands were subjected to *A.*

lumbricoides β-tubulin PCR. Seventeen samples from Guatemala yielded useable sequence fragments. At the time of writing, thirteen samples had been sequenced and analyzed and all were homozygous sensitive at codons 167, 198, and 200. Due to problems with DNA degradation with the Solomon Islands samples and the low sensitivity of even the nested β-tubulin PCR (single copy gene), only one acceptable β-tubulin fragment by next generation sequencing. This sample was also homozygous sensitive. A repeated internal control was also sequenced for *A. lumbricoides* from the same positive control sample, taken from the DNA of a whole adult worm, that had been through multiple rounds of PCR was included and yielded an identical sequence in all cases.

Three genetically distinct alleles were identified in a (non-coding) intron sequences in the β-tubulin gene. The single positive Solomon Islands sample shared one of these alleles with an identical sequence. One of the Guatemala specimens was contained two separate genotypes of this allele. Within this specimen, one allele was present at a proportion of 8.1% of sequencing reads and the second allele was found in 91.9% of sequencing reads. Were seen between samples, and one sample possessed three different non-expressed intron genotypes demonstrating that the sequences generated were variable, though none varied at the expressed codons associated benzimidazole resistance.

We were unable to include the results from the *N. americanus* samples from either population due to time constraints. However, the method that we have proposed here is sound and can be used to obtain results of this kind for both *N. americanus* and other species of interest in the future.

Solomon Islands: Factors associated with STH infection

A statistically significant association between sex and *A. lumbricoides* infection was found, with more infections occurring among women than men (OR=1.42, 95% CI=1.03, 1.90) (Table 3). For both hookworm (OR=0.86, 95% CI=0.59, 1.25) and *T. trichiura* (OR=2.20, 95% CI=0.83, 5.86) infections, we did not find any association between sex and parasitic infection was found at a p-value of 0.05 (Table 3). For all three parasites of interest, statistically significant associations were found between village and infection prevalence. Ngongosila had the highest infection prevalence (55.4%) of *A. lumbricoides* and had the largest number of hookworm infections (n=67); however, the highest hookworm infection prevalence was found in Na'au village (n=6, %=35.3%) (Table 3). Using Ngongosila as the reference group, we found that residents of Kwai village had 50% lower odds of having an *A. lumbricoides* infection (95% CI= 0.36, 0.68) (Table 3). We did not find an association between odds of infection and residence in Na'au village for *A. lumbricoides*. For hookworms, again using Ngongosila as the reference group, we found that residents of Kwai village had 58% lower odds of having a hookworm infection (95% CI= 0.40, 0.86) (Table 3). Finally, for *T. trichiura*, we found that residents of Kwai village had 17% lower odds of infection compared to residents of Ngongosila village (95% CI= 0.02, 0.37) (Table 3).

Using the 0-10-year-old age group as the reference group, the odds of *A. lumbricoides* infections all decreased significantly as age increased by one 10-year age group (Table 4). The lowest odds of infection occurred in the 61+ age group (OR=0.15, 95% CI=0.05, 0.39) while the 11-20-year-old age group did not differ significantly from the reference group (OR=0.97, 95% CI=0.64, 1.48) (Table 4). The opposite trend was

seen in hookworms, with the odds of infection decreasing as age increased by one 10-year age group (Table 4). The highest odds of infection occurred in the 51-60-year-old age group compared to the reference group (OR=15.0, 95% CI=6.2, 36.2) while the lowest odds of infection compared to the reference group occurred in the 21-30-year-old age group (OR=3.93, 95% CI=1.83, 8.41) (Table 4). Still, odds of infection in all age groups differed significantly when compared to the odds of infection in the reference group (Table 4). Finally, there was no evidence of statistical difference between *T. trichiura* infection and age; however, this may be due to the relatively low numbers of *T. trichiura* infections found in the study population.

Line plots were produced for Ngongosila and Kwai villages for both *A. lumbricoides* and hookworm infections; Na'au village had too few observations to see a trend in graphical form. *A. lumbricoides* infections both followed the expected trend of high prevalence in pre-school aged and school-aged children and low prevalence among adults in both villages (Figure 1). However, hookworm infections did not follow the expected trend of low prevalence in children and high prevalence in adults in either village (Figure 2). Rather, prevalence of hookworms seemed fairly equal throughout all age groups (Figure 2). More research is required to elucidate why hookworm transmission is different in these communities compared to other areas of the world.

Infection intensity, measured as eggs per gram, was also recorded during the initial study and further explored here to determine the relationship between age and burden of disease. For *A. lumbricoides* infection, the odds of a low intensity infection increase significantly among 11-20-year olds compared to children in the 0-10-year-old age group (OR= 1.96, 95% CI=1.19, 3.24) (Table 5). For all older age groups, no association was

found between low intensity infection and age. Contrastingly, all age groups had a statistically significant decrease in odds of medium intensity infection for *A. lumbricoides* compared to the 0-10-year-old age group (Table 5). No association was found among the high intensity age group; although, there were overall much fewer high intensity infections than low intensity infections. For hookworms and *T. trichiura*, odds ratios were only calculated for low intensity infections due to the dearth of medium or high intensity infection. However, when compared to the 0-10 age group, all older age groups had significantly higher odds of low intensity hookworm infection (Table 5). No association was found between age and low intensity infection for *T. trichiura* (Table 5).

Guatemala: Factors associated with STH infection

No association was found between sex and infection with either *A. lumbricoides* or *T. trichiura* (Table 6). Still, there was a significant association between STH infection and site of admission to the study, evidence that perhaps people who choose to receive their health care from one type of facility may differ in risk factors from people who choose to attend a different type of facility (Table 6). For *A. lumbricoides*, participants enrolling at all three admission sites had a high prevalence of infection, and people who enrolled at hospitals had significantly reduced odds of infection compared to those who enrolled at health facilities (OR=0.28, 95% CI=0.11, 0.73) (Table 6). Similarly, for *T. trichiura*, a statistically significant association was found between facility type and infection, with participants enrolling in hospitals having the highest prevalence (45.9%) upon entrance into the study (OR=4.20, 95% CI=1.81, 9.73) (Table 6). There was no association found between 10-year-age-group and infection, even though we would expect such an association to be present based on previous literature (Table 7).

To further investigate the site-infection relationship, age line plots were produced for *A. lumbricoides* (Figure 3). All three panels to show that infection follows the expected trend, high prevalence in the very young and low prevalence above school-aged children. In panel (C), cases brought into the hospital tend to be very young infants and then spikes again around pre-school age with little other variation in the rest of the plot (Figure 3). Panels (A) and (B), health facilities and health posts, respectively, follow the same trend and have more variation and a wider range of ages (Figure 3). This could indicate the types of healthcare that people in the sampled areas prefer to visit for treatment of diarrheal illness. Likewise, line plots were produced to further investigate the association between site of admission and *T. trichiura* infection (Figure 4). The *T. trichiura* infections followed essentially the same pattern as the *A. lumbricoides* infections in Figure 3; however, hospitals had the widest age range of the three admission site types for *T. trichiura* positive participants (Figure 4).

DISCUSSION

Through a novel next generation sequencing analysis approach, we did not find any resistant SNPs from either the Solomon Islands or Guatemala samples. It is not surprising that we did not find any resistant SNP in *A. lumbricoides* due to the suspected low prevalence of resistance in human populations. Still, most individual samples that were sequenced returned only one genotype of worm, which is unexpected due to the ease of continuous transmission among these communities. We did not find any evidence of resistant SNPs in the *N. americanus* samples, despite expecting to find some evidence of resistance in this population. Despite the sound method of obtaining information of SNPs at the β-tubulin gene that we propose here, we were unable to include those results in this iteration.

One finding of this study is that women in the Solomon Islands have significantly higher odds of *A. lumbricoides* infection compared to men. This difference could be due to the division of labor between males and females; women may be working more in the soil for gardening and farming while the men typically do more of the fishing and hunting (14). Neither hookworm nor *T. trichiura* infections were associated with age. However, village and STH infection were significantly associated across all three STH of interest. Kwai village consistently had significantly lower odds of infection for *A. lumbricoides*, *T. trichiura*, and hookworms compared to Ngongosila village. This finding is interesting because the lifestyle, approach to hygiene and sanitation, and environment of these two different villages are not dissimilar. Further research is needed to elucidate why there should be lower odds of infection of these STH in Kwai but not the nearby Ngongosila.

An interesting result of this study is that, in addition to a simple association between 10-year-age-groups and *A. lumbricoides* infections, we found a relationship between *A. lumbricoides* infection intensity and age. We found that as age increases by 10 years, the odds of low intensity infection increases compared to the 0-10-year-old age group. Conversely, as age increases by 10 years, the odds of medium or high intensity infection decreases. Thus, pre-school and school-aged children are not only the most at risk for *A. lumbricoides* infection overall, but they are also more susceptible to medium and high intensity infection which are more likely to have serious health consequences. There is also an association between hookworm infection and 10-year-age groups. More interestingly, as age increases by 10 years, odds of low intensity hookworm infection increases significantly compared to 0-10-year-olds. This relationship was particularly significant among 51-60-year-old participants. This pattern fits with the anticipated relationship between age and hookworm infection and supports the body of evidence that the majority of hookworm infections are low burden (4; 6; 13). As such, in the Solomon Islands study there were too few cases of medium or high intensity cases to calculate meaningful odds ratios. For *T. trichiura*, we found no association among low intensity infections and lacked any data on medium or high intensity cases. *T. trichiura* is not as prevalent in this region, but perhaps if we had expanded our sample size we would have seen an association similar to *A. lumbricoides* as we would expect from the literature (10). One way to interrupt the chain of transmission between children and adults, and adults back to children, would be to scale-up MDA efforts to include the whole community and not just pre-SAC and SAC as seen in other studies and mathematical simulations (4; 9).

In the Guatemala study, site of admission and STH infection were found to be significantly associated for both *A. lumbricoides* and *T. trichiura*. Despite the similarity in infection patterns between these two species, we found that their associations between admissions to hospitals versus health centers were reversed. Compared to people being admitted to health centers, people who were admitted to hospitals had significantly decreased odds of *A. lumbricoides* infection. Conversely, compared to people admitted to health facilities, people who were admitted to hospitals had significantly increased odds of *T. trichiura*. These differences are unlikely to be due to differences between the sample areas of Santa Rosa and Quetzaltenango because there were relatively few observations from Quetzaltenango in this analysis (n=8) and participants from this subset were evenly admitted into all three different health facility site types. Therefore, we propose that perhaps very young children (ages 0-4) have worse health outcomes from *T. trichiura* infection than *A. lumbricoides* infection that warrant a trip to the hospital rather than a community health center.

One strength of this study is the use of an entirely novel Next Generations Sequencing to identify β-tubulin SNPs. Next Generation sequencing allows us to investigate SNPs from every single separate STH egg DNA that was amplified, not just the most prevalent genotype in each stool sample. Thus, if a β-tubulin resistance allele exists in our study populations, then we would have a good chance of finding it. To be even more certain that the sequencing results that we obtained are valid, we sequenced repeats of the same positive control that had been used in subsequent PCRs to obtain a rough estimate of the inherent variability of our sequencing reaction.

Limitations of this study is that we were unable to do more in-depth statistical analysis with Guatemala study due to lack of the full dataset; we only used those participants who had at least one STH infection for our analysis. Also, these people who had an STH infection may be systematically different from other people in the area. These people may be more likely to seek out medical care due to education or socioeconomic status. Furthermore, we lacked concrete MDA records for both study populations. Therefore, we can only make informed conjectures about the amount of preventative chemotherapy that individuals in these communities have received. Still, we can be fairly certain that the participants from the Solomon Islands are naïve to MDA efforts while we know that the participants from Guatemala have received MDA in the form of ivermectin as part of schistosomiasis elimination programs. As such, the existing STH populations in the Guatemala participants was already exposed to certain selection pressures even if these pressures did not act on the β-tubulin genes.

We experienced difficulties with low primer binding sensitivity and needed to alter the Thermocycler program several times. Any positive that we found on gel electrophoresis was sent to be sequenced, but even with our optimized nested PCR, relatively few samples out of the larger dataset were acceptable for sequencing. However, we were successfully able to optimize nested PCR reactions for *A. lumbricoides* and both fragments of *N. americanus*. Due to time constraints, we ultimately did not have time to fully optimize the reaction for *Anclyostoma* spp. and *T. trichiura* due to poor sensitivity even in nested PCR. In spite of these difficulties, we are confident in the quality of our results due to the use of an internal repeated positive control. One final limitation of this study was the age of DNA samples. Some of our

oldest samples were collected and frozen since 2007 and could have become degraded over time. This issue could possibly explain some of the difficulties that we experienced with creating high sensitivity primers to isolate the β-tubulin genes for *T. trichiura* and the *Ancylostoma* spp. However, we are still confident in the quality of our sequencing results based on high DNA yield following PCR quantified through Nanodrop.

Conclusion, Future Directions, and Public Health Implications

This study is the first of its kind to use conventional nested PCR and Next Generation sequencing to isolate species-specific β-tubulin genes in order to identify anthelmintic resistance to benzimidazoles. Due to time constraints, we were unable to fully optimize nested PCR reactions for *T. trichiura* and the *Ancylostoma* spp.; however, we were able to optimize nested PCR reactions for *A. lumbricoides* and both fragments of *N. americanus*. It is important that we were able to optimize these two STH species before the others for several reasons. First, *A. lumbricoides* and *N. americanus* were the most prevalent STH in the study populations used in this study. Second, Diawara et al. has already provided evidence for SNPs at the β-tubulin gene for *T. trichiura* from populations in Panama, Haiti, and Kenya (42). Any polymorphism found from *A. lumbricoides* and *N. americanus* would have major public health implications; therefore, other studies using our methods should be conducted to test for replicability in other situations. While we are confident that our primers are suitable to be used in future studies of this kind, more work needs to be done to find better and quicker ways of isolating the β-tubulin gene from STH through molecular techniques. Furthermore, we suggest that individual institutions and governments should retain preventative chemotherapy records at the population level, at least, in order to inform further MDA

policy changes should we observe an increase in benzimidazole resistance. Lessons learned from the devastating consequences of the overuse of benzimidazoles in veterinary medicine should inform our urgency to conduct routine surveillance of this kind (28). Any sign of benzimidazole resistance should be taken seriously as it may herald the necessity to revolutionize how we perform MDA campaigns worldwide (27; 42; 53). Additionally, our analysis of infection prevalence and demographic data revealed several interesting associations between age, in the Solomon Islands, and site of admission, in Guatemala. We must put more effort into stopping the chain of transmission in endemic locations such that preventative chemotherapy is no longer so heavily relied upon through other sanitation and hygiene initiatives.

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TABLES

Table 1. Demographic characteristics and infection prevalence of study populations from the Solomon Islands (2014) and Guatemala (2007-2016)

Solomon Islands	Number of participants, (%)		**Guatemala**	Number of participants, (%)	
Sex			**Sex**		
Male	340	(50.1)	Male	89	(46.6)
Female	338	(49.9)	Female	102	(53.4)
Infection			**Infection**		
A. lumbricoides	299	(80.4)	*A. lumbricoides*	162	(84.8)
T. trichiura	4	(1.08)	*T. trichiura*	29	(15.2)
Hookworms	69	(18.5)			
Village			Site of Admission		
Ngongosila	271	(40.3)	Hospital	37	(19.4)
Kwai	384	(57.1)	Health Post	59	(30.9)
Na'au	17	(2.53)	Health Facility	95	(49.7)
	Mean	**SD**		**Mean**	**SD**
Age (years)	23.5	19.1	**Age (years)**	9.8	15.1
	# patients in age group, (%)			**# patients in age group, (%)**	
0-10	220	(32.4)	<0	6	(3.14)
11-20	148	(21.8)	0-10	142	(74.4)
21-30	91	(13.4)	11-20	13	(6.82)
31-40	96	(14.1)	21-30	16	(8.38)
41-50	61	(8.97)	31-40	3	(1.57)
51-60	33	(4.85)	41-50	1	(0.52)
61+	31	(4.56)	51-60	4	(2.09)
			61+	6	(3.14)

Table 2. Internal and external primers used in conventional nested-PCR for species-specific β-tubulin genes

Species	**Codons Included**	**Primary PCR Primers**	**Nested PCR Primers**
A. lumbricoides	167, 198, 200	F: CTCGCTTGGTGGAGGCAC	F: TCCGTGAAGAATACCCCGACA
		R: TCCCATTCCAGCTCACAGACG	R: CAGATGTCGTACAAAGCCTCATT
N. americanus	167	F: CGAAGGGGCACTACACTGAA	F: GTGACTGTCTCCAGGTAATTCG
		R: CCCATAGCGTCGAAACCACTA	R: CTATAACGTACCTTTGGCGAGGG
	198, 200	F: GGGCGCGTATTCTTTTCGAC	F: GTTTCCGACACTGTGGTTGAGC
		R: AGCGAAACCAGGCATGAAGA	R: GAGTTCGTTACTAGCCAGCTCACC

Table 3. Association between population demographics and soil-transmitted helminth infection in the Solomon Islands, 2014

	# infected, (%)		Total	Odds Ratio	95% CI	
A. lumbricoides						
Gender*						
Female	163	(47.9)	340	1.42	1.03	1.90
Male	134	(39.6)	338	1.00		
Village						
Ngongosila	150	(55.4)	271	1.00		
Kwai*	146	(38.0)	384	0.50	0.36	0.68
Na'au	0	(0.0)	17	-	-	-
Hookworms (*N. americanus & A. ceylanicum*)						
Gender						
Female	64	(18.8)	340	0.86	0.59	1.25
Male [a]	72	(21.3)	338	1.00		
Village						
Ngongosila [a]	67	(24.7)	271	1.00		
Kwai*	62	(16.1)	384	0.59	0.40	0.86
Na'au	6	(35.3)	17	1.66	0.59	4.66
Trichuris trichiura						
Gender						
Female	13	(3.82)	340	2.20	0.83	5.86
Male [a]	6	(1.78)	338	1.00		
Village						
Ngongosila [a]	16	(5.90)	271	1.00		
Kwai*	2	(0.52)	384	0.83	0.02	0.37
Na'au	0	(0.0)	17	-	-	-

[a] reference group
* statistically significant at p=0.05

Table 4. Odds of soil-transmitted helminth infection among total study population by 10-year age categories in the Solomon Islands, 2014

Age (yrs)	# infected, (%)		Total	OR	95% CI	
A. lumbricoides*						
0-10[a]	125	(56.8)	220	1.00		
11-20	83	(56.1)	148	0.97	0.64	1.48
21-30*	32	(35.2)	91	0.41	0.25	0.68
31-40*	21	(21.9)	96	0.21	0.12	0.37
41-50*	23	(37.7)	61	0.46	0.26	0.82
51-60*	10	(30.3)	33	0.33	0.15	0.73
61+*	5	(16.1)	31	0.15	0.05	0.39
Hookworms*						
0-10[a]	13	(5.91)	220	1.00		
11-20*	37	(25.0)	148	5.31	2.71	10.4
21-30*	18	(19.8)	91	3.93	1.83	8.41
31-40*	27	(28.1)	96	6.23	3.05	12.7
41-50*	16	(26.2)	61	5.66	2.54	12.6
51-60*	16	(48.5)	33	15.0	6.2	36.2
61+*	9	(29.0)	31	6.51	2.5	17.0
T. trichiura						
0-10[a]	8	(3.64)	220	1.00		
11-20	6	(4.05)	148	1.12	0.38	3.30
21-30	3	(3.30)	91	0.90	0.23	3.50
31-40	0	(0.00)	96	0.00	-	-
41-50	1	(1.64)	61	0.44	0.05	3.60
51-60	1	(3.03)	33	0.83	0.10	6.80
61+	0	(0.00)	31	0.00	-	-

[a] reference group
* statistically significant at p=0.05

Table 5. Odds of low, medium, or high intensity infection from *A. lumbricoides*, hookworms, or *T. trichiura* infections in the Solomon Islands, 2014 by 10-year age categories

A. lumbricoides						Hookworms (*N. americanus* & *A. celynicum*)						*T. trichiura*					
	# Low Intensity, (%)	**Total**	**OR**	**95% CI**			**# Low Intensity, (%)**	**Total**	**OR**	**95% CI**			**# Low Intensity, (%)**	**Total**	**OR**	**95% CI**	
0-10 [a]	37 (16.8)	220	1.00			0-10 [a]	12 (5.45)	220	1.00			0-10 [a]	8 (3.64)	220	1.00		
11-20*	42 (28.4)	148	1.96	1.19	3.24	11-20*	37 (25.0)	148	5.78	2.90	11.5	11-20	6 (4.05)	148	1.12	0.38	3.30
21-30	22 (24.2)	91	1.58	0.87	2.85	21-30*	18 (19.8)	91	4.27	1.96	9.30	21-30	3 (3.30)	91	0.90	0.23	3.48
31-40	16 (16.7)	96	0.99	0.52	1.89	31-40*	25 (26.0)	96	6.10	2.91	12.8	31-40	0 (0.00)	96	-	-	-
41-50	17 (27.9)	61	1.91	0.99	3.70	41-50*	16 (26.2)	61	6.16	2.72	13.9	41-50	1 (1.64)	61	0.44	0.05	3.60
51-60	6 (18.2)	33	1.10	0.42	2.85	51-60*	16 (48.5)	33	16.3	6.65	40.0	51-60	1 (3.03)	33	0.83	0.10	6.84
61+	4 (12.9)	31	0.73	0.24	2.22	61+*	7 (22.6)	31	7.09	2.69	18.7	61+	0 (0.00)	31	-	-	-
	# Med Intensity, (%)	**Total**	**OR**	**95% CI**			**# Med Intensity, (%)**	**Total**									
0-10 [a]	80 (36.4)	220	1.00			0-10	0 (0.00)	220									
11-20*	37 (25.0)	148	0.58	0.37	0.93	11-20	1 (0.68)	148									
21-30*	9 (9.89)	91	0.19	0.09	0.40	21-30	0 (0.00)	91									
31-40*	5 (5.21)	96	0.10	0.04	0.25	31-40	1 (1.04)	96									
41-50*	6 (9.84)	61	0.19	0.08	0.46	41-50	0 (0.00)	61									
51-60*	4 (12.1)	33	0.24	0.08	0.71	51-60	0 (0.00)	33									
61+*	1 (3.23)	31	0.06	0.01	0.44	61+	0 (0.00)	31									
	# High Intensity, (%)	**Total**	**OR**	**95% CI**			**# High Intensity, (%)**	**Total**									
0-10 [a]	8 (3.64)	220	1.00			0-10	1 (0.45)	220									
11-20	4 (2.70)	148	0.74	0.22	2.49	11-20	0 (0.00)	148									
21-30	1 (1.10)	91	0.29	0.04	2.40	21-30	0 (0.00)	91									
31-40	0 (0.00)	96	-	-	-	31-40	1 (1.04)	96									
41-50	0 (0.00)	61	-	-	-	41-50	0 (0.00)	61									
51-60	0 (0.00)	33	-	-	-	51-60	0 (0.00)	33									
61+	0 (0.00)	31	-	-	-	61+	0 (0.00)	31									

[a] reference group
* statistically significant at p=0.05

Table 6. Association between population demographics and soil-transmitted helminth infection in Quetzaltenango and Santa Rosa, Guatemala, 2007-2016

	# infected,	(%)	Total	Odds Ratio	95% CI	
A. lumbricoides						
Sex						
Female	91	(89.2)	102	2.10	0.93	4.72
Male [a]	71	(79.8)	89	1.00		
Site of Admission						
Hospital*	26	(70.3)	37	0.28	0.11	0.73
Health Facility [a]	85	(89.5)	95	1.00		
Health Post	51	(86.4)	59	0.75	0.28	2.02
T. trichiura						
Sex						
Female	19	(18.6)	102	0.53	0.27	1.03
Male [a]	27	(30.3)	89	1.00		
Site of Admission						
Hospital*	17	(45.9)	37	4.20	1.81	9.73
Health Facility [a]	13	(13.7)	95	1.00		
Health Post	16	(27.1)	59	1.40	0.62	3.16

[a] reference group
* statistically significant at p=0.05

Table 7. Association between soil-transmitted helminth infection among total study population by 10-year age categories in Quetzaltenango and Santa Rosa, Guatemala, 2007-2016

Age (yrs)	# infected,	(%)	Total	Odds Ratio	95% CI	
A. lumbricoides						
<0	6	(100.0)	6	-	-	-
0-10 [a]	124	(87.3)	142	1.00		
11-20	9	(69.2)	13	0.33	0.09	1.17
21-30	12	(75.0)	16	0.44	0.13	1.50
31-40	2	(66.7)	3	0.29	0.03	3.37
41-50	1	(100.0)	1	-	-	-
51-60	4	(100.0)	4	-	-	-
61+	4	(66.7)	6	0.29	0.05	1.70
T. trichiura						
<0	0	(0.0)	6	-	-	-
0-10 [a]	33	(23.2)	142	1.00		
11-20	4	(30.8)	13	1.47	0.43	5.08
21-30	5	(31.3)	16	1.50	0.49	4.63
31-40	1	(33.3)	3	1.65	0.15	18.8
41-50	0	(0.0)	1	-	-	-
51-60	1	(25.0)	4	1.10	0.11	10.9
61+	2	(33.3)	6	1.65	0.29	9.42

[a] reference group

FIGURES

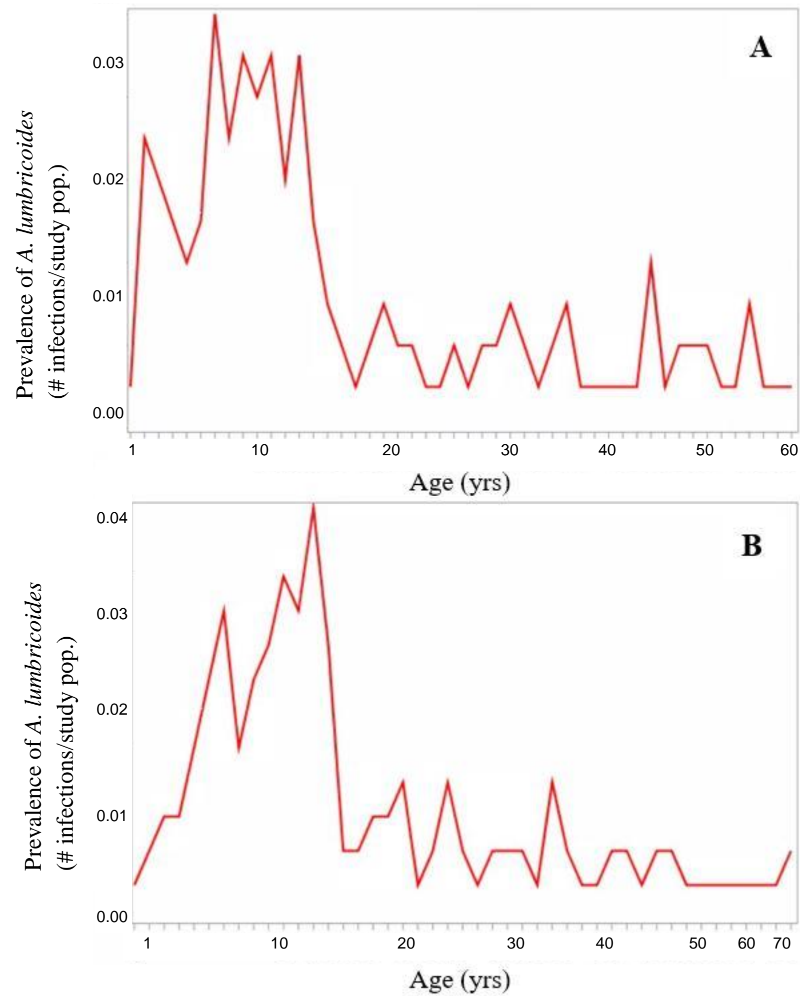

Figure 1. Prevalence of *Ascaris lumbricoides* infections by age in years in study population in the Solomon Islands, 2014 by village. In panel (A), participants from Ngongosila village. In panel (B), participants from Kwai village.

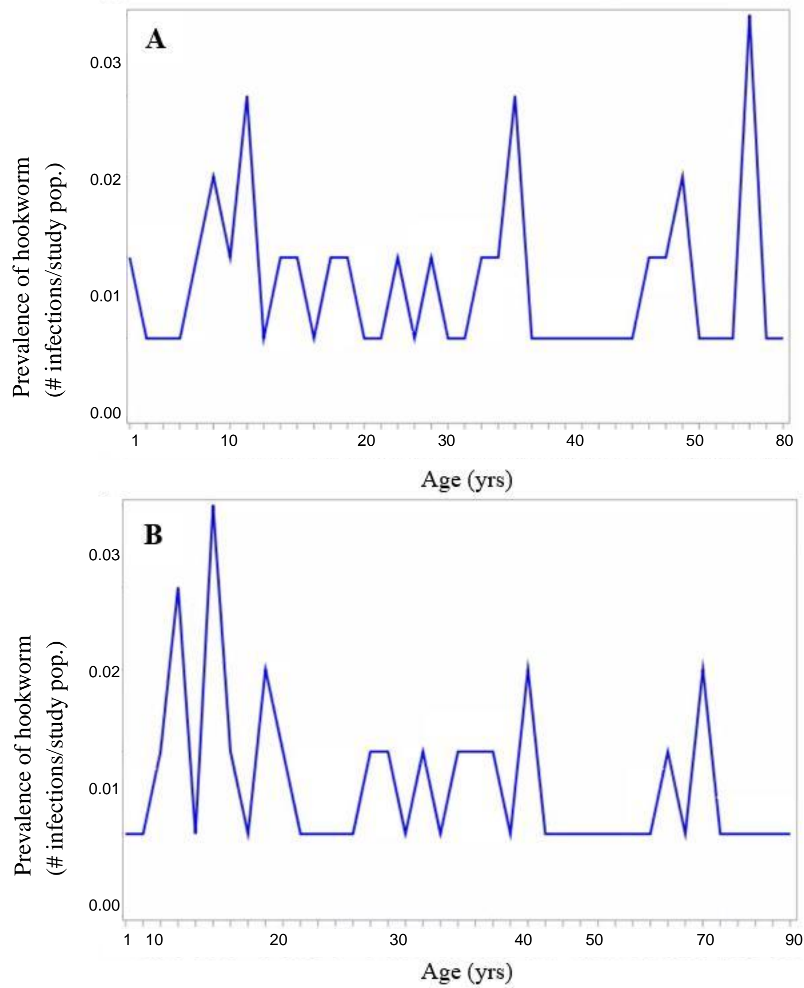

Figure 2. Prevalence of hookworm infections (including *Necator americanus* and *Ancylostoma* spp.) by age in years in study population, in the Solomon Islands 2014, by village. In panel (A), participants from Ngongosila village. In panel (B), participants from Kwai village.

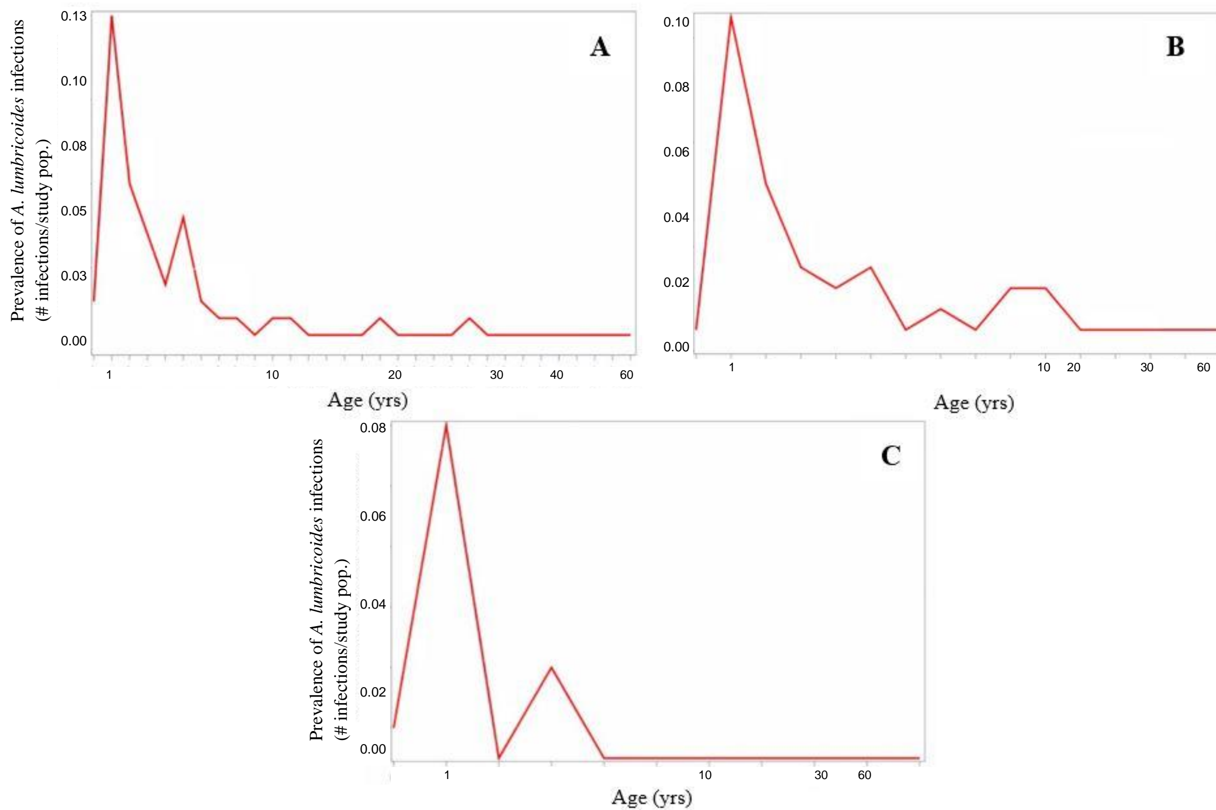

Figure 3. Prevalence of *Ascaris lumbricoides* infections by age in years in study population from Quetzaltenango and Santa Rosa, Guatemala, 2007-2016, by site of admission into the study. Panel (A): participants admitted from community health centers. Panel (B): participants admitted from community health outposts. Panel (C): participants admitted from hospitals.

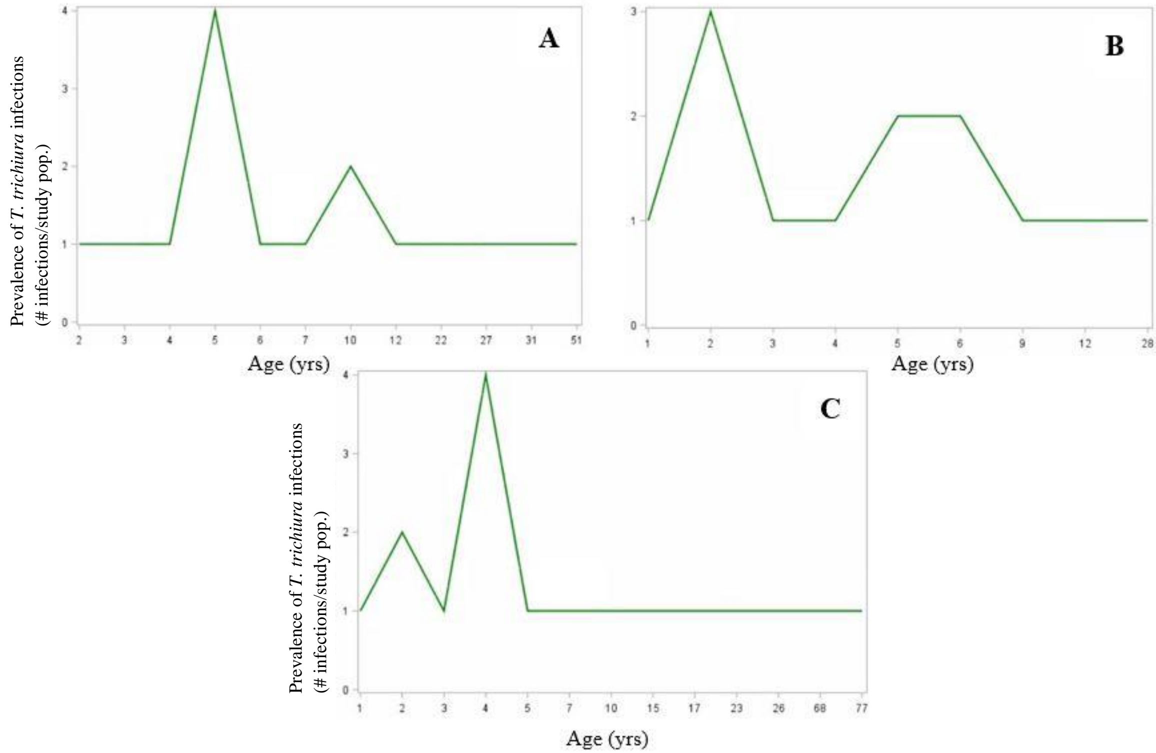

Figure 4. Prevalence of *Trichuris trichiura* infections by age in years in study population from Quetzaltenango and Santa Rosa, Guatemala, 2007-2016, by site of admission into the study. Panel (A): participants admitted from community health centers. Panel (B): participants admitted from community health outposts. Panel (C): participants admitted from hospitals.

APPENDIX A

Qiagen DNeasy Powersoil Kit - Instructions

Prep Step 1. Turn on the heat block to 60ºC.

Prep Step 2. Find out whether samples are fixed or preserved. If so, the fixative or preservative must be removed and and equilibrated O/N (jump to Procedure Step 1.)

Prep Step 3. Replace "powerbeads" with silica-zirconia beads.

Instructions: Pull number of powerbead tubes from the kit which will be needed for DNA extraction (i.e., 12 samples = 12 tubes.) and place in a rack. THESE WILL BE USED AS 2 mL screw cap tube. Pull off supernatant (about 3-400 uL) and pool in a 15 mL conical tube for later use. Replace beads (approx. to the 0.5 mark, see reference of 500uL.) with silica-zirconia beads. DO NOT THROW AWAY TUBES OR CAPS. Add 370 uL lysis solution (pooled in the 15 mL conical tube)

Prep Step 4. Check that all buffers contain enough volume for run. Use buffers with least volume first. CHECK SOLUTION C1 to ensure it is dissolved. If precipitate present, heat on heating block until completely dissolved at 60ºC. Once complete, turn heating block to 65ºC.

Procedure Step 1: If samples are preserved or fixed, add sample to 1.7mL tube and fill to 1 mL. Centrifuge the sample for 3 min at 3000xG. Remove ALL supernatant and add 1mL sterile saline and vortex for 5 sec. Leave O/N at 4ºC.

The following day, centrifuge at 3000xG for 3 min and remove all supernatant. Store at 4ºC. DNA MUST BE EXTRACTED within 12 hours.

Procedure Step 2: Label tubes and place in tube rack. Place approximately 0.2 mL or 200mg of feces into empty 1.7Ml tube and resuspend in diH2O to the 1.5Ml mark. Vortex homogenize on HIGH for 1 min. Centrifuge at 3000Xg for 3 min. Remove supernatant.

Procedure Step 3: Add 60uL of solution C1 to bead tubes + lysis buffer. Place 0.2 mL feces from step 2 into each corresponding tube.

Procedure Step 4: Heat tubes containing sample+beads+C1 for 10 minutes at 65ºC. During this 10 minutes, prepare and label tubes for subsequent steps.

Procedure Step 5: Bead beat for 3 min on high speed. Allow samples to cool 5 minutes to RT. Centrifuge at 10000xg for 1 min. During the minute, add 250uL of C2 solution to clean 2 mL collection tube from kit.

Procedure Step 6: Transfer approx. 400-500uL supernatant to tubes containing 250 uL C2 solutions and vortex 5sec. Incubate at 4ºC for 5 minutes. Centrifuge at 10000xg for 1 min. During the 1 minute incubation, add 200uL C3 solution to clean tubes.

Procedure Step 7: Transfer 600uL supernatant from step 6 to tubes containing 200uL C3. Avoid pellet. DO NOT transfer more than 600uL. Vortex for 5s and incubate at 4ºC for 5 min.

Centrifuge at 10000xg for 1 min. During the 1 min incubation, SHAKE SOLUTION C4 and add 1200uL of solution C4 to clean tubes.

Procedure Step 8: Transfer up to 750uL supernatant to tubes containing 1200uL C4 solution and vortex for 5 sec.

Procedure Step 9: Load approximately 675 (650uL is ideal) of C4+supernatant from step 8 onto a spin column filter. Centrifuge at 10000xg for 1 min. Discard flow through. Repeat process with an additional 650uL. Repeat step until all sample volume is consumed (~3 times.) You can continue to use the same collection tube.

Procedure Step 10: Add 500uL C5 solution and centrifuge for 30s at 10000xg using the same collection tube. Discard flow through. Centrifuge to dry column for 1 minute at 10000xg using the same collection tube. During one minute step, label new collection tubes for elution and storage.

Procedure Step 11: Place spin column in fresh collection tube. Add 50 uL elution buffer C6 to filter.

Note: If performing DNA extraction for UPDx process, use DiH2O as elution buffer so that C6 elution buffer does not affect downstream applications.

Procedure Step 12: Centrifuge for 30s at 10000xg. Discard spin filter. Store at -20ºC.