MOLECULAR AND EPIDEMIOLOGICAL STUDIES ON HUMAN SOIL-TRANSMITTED HELMINTHS BEFORE AND AFTER ALBENDAZOLE TREATMENT

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I dedicate this thesis to my mother Suzanne Diawara, my sister Fatou Diawara and in memory of my father Dr. Alpha Ibrahima Diawara, and my grandmother Adja Condé.

"There is only one thing that makes a dream impossible to achieve: the fear of failure"

Paulo Coelho

ABSTRACT

Background: Soil transmitted helminths (STHs) are gastrointestinal nematodes of humans. The roundworm *Ascaris lumbricoides,* the whipworm *Trichuris trichiura,* and the hookworms *Ancylostoma duodenale* and *Necator americanus,* remain highly prevalent in most tropical countries of the developing world. Infections with STHs affect over 2 billion people worldwide causing many disabilities and death. Periodic deworming with anthelmintic (AH) drugs provided at the community-level is the principal means of controlling STH infections. Drug treatments consist of mainly two drugs, albendazole (ABZ) and mebendazole (MBZ) that belong to the benzimidazole (BZ) class of AHs. However, repeated treatment with BZs can cause selection of mutations in the β-tubulin gene leading to resistance. In veterinary nematodes, resistance is commonly associated with a single nucleotide polymorphism (SNP) in the β-tubulin gene at codon 200 causing an amino acid change from phenylalanine to tyrosine. In the whipworm *T. trichiura* the same genetic change has been identified in samples from treated and untreated subjects in Panama and Kenya. In the meantime, reduced or lowered ABZ and MBZ efficacies have been widely reported in *T. trichiura* infected patients and raise a concern with respect to the possible development of BZs resistance. To maintain an effective control strategy it is crucial to identify resistance, if any, at an early stage. Based on veterinary parasites, SNPs 167 and 198 result in amino acid changes from phenylalanine to tyrosine and from glutamate to alanine, respectively, which have also been associated with BZs

resistance. **Method:** In this regard, we have developed accurate molecular assays for STHs to detect β-tubulin genetic changes at positions 200, 167 and 198 that are associated with resistance. We also optimized the *in vitro* egg hatch assay (EHA) for canine hookworms and have applied it to human hookworms for assessing the response to BZs. Both assays have been tested on samples collected in endemic countries. Furthermore we carried out a 14-month longitudinal study in Haiti to investigate the response of STHs to two rounds of ABZ treatment in 344 subjects from five endemic communities naive for ABZ. Stool samples were collected before ABZ treatment, and then two-weeks and two-months after the first ABZ round and then just prior to and two-weeks after a second round of ABZ treatment. **Results**: In this study, genetic markers have been validated with control plasmids for position 200 in hookworms, also for positions 167 and 198 in hookworms, *A. lumbricoides* and *T. trichiura*. We have confirmed that our method can be used to discriminate between susceptible and resistance genotypes. In cross sectional studies involving samples collected in Haiti, Kenya and Panama, we identified at low frequency, before and after treatment, the resistance-associated SNP at codon position 200 in hookworms from Kenya, while the egg reduction rate (ERR) values indicated good drug efficacy. In *A. lumbricoides,* a SNP at position 167 was identified at high frequency before and after one drug treatment of subjects in Haiti, Kenya and Panama while ABZ efficacy remained high. In *T. trichiura,* the SNP at codon position 200 was detected and there was a significant increase in the homozygous resistance-type from 51.3% to 67.8% in Kenya and 3.1% to 55.3% in Haiti after one round of ABZ treatment. The

associated drug efficacies, based on faecal egg counts (FEC), were low in Kenya (10.1%) and moderate in Haiti (49.7%). From this result in Haiti, we classified subjects infected with *T. trichiura* into good, intermediate or poor ABZ responders. The frequency of SNPs 198 and 200 increased significantly after one round of ABZ treatment in the intermediate and poor response groups. Also, a persistence of the parasite and resistance alleles were found both two months and one year after treatment of subjects classified at baseline as intermediate and poor responders. **Conclusion:** This study has shown for the first time evidence of an association between genetic changes in the β-tubulin gene of *T. trichiura* and poor ABZ efficacy against this parasite.

ABRÉGÉ

Contexte: Les géohelminthes sont des nématodes parasitant le système gastrointestinal de l'Homme. La prévalence du vers rond *Ascaris lumbricoides*, du trichocéphale *Trichuris trichiura* et des ankylostomes est élevée dans la majorité des régions tropicales des pays en voie de développement. Dans le monde, plus de 2 milliards de personnes sont atteintes de géohelminthiase, causant de nombreux handicaps et parfois même le décès de ces personnes. Pour lutter contre les géohelminthiases une des stratégies consiste à déparasiter périodiquement des communautés endémiques avec des antiparasitaires. Les traitements médicamenteux reposent principalement sur l'utilisation de l'albendazole (ABZ) et du mébendazole (MBZ), appartenant à la famille des benzimidazoles (BZ). Cependant, l'utilisation répétitive de BZ peut causer une sélection au niveau du gène de la β-tubuline et entraîner une résistance. Chez les nématodes parasitant le bétail, la résistance est fréquemment due à un polymorphisme d'un nucléotide (SNP) au codon 200 de la β-tubuline, causant une substitution de l'acide aminé phénylalanine par la tyrosine. Chez le trichocéphale *T. trichiura* le même changement génétique a été identifié dans des échantillons collectés au Kenya et au Panama auprès de personnes sous traitement à l'ABZ ou non. Parallèlement, de nombreuses études ont rapportés une faible efficacité ou une efficacité réduite à l'ABZ chez *T. trichiura*, ce qui a soulevé une inquiétude quant au développement de la résistance. Pour maintenir une stratégie de contrôle efficace, il est indispensable de détecter rapidement la résistance, si présente. Les SNPs 167 et

198 causant une substitution de la phenylalanine à la tyrosine et du glutamate à l'alanine, ont aussi été associés à la résistance chez les parasites infestant le bétail. **Méthode** : Dans ce contexte, nous avons développé pour les géohelminthes des tests moléculaires précis pour détecter les changements génétiques de la β-tubuline aux codons 167, 198 et 200, associés à la résistance. Nous avons aussi optimisé un test in vitro, le EHA, chez les ankylostomes infestant la race canine. Puis nous l'avons testé sur des ankylostomes infestant l'Homme pour déterminer la réponse aux BZs. Les deux tests (moléculaire et *in vitro*), ont été expérimentés sur des échantillons collectés dans des pays endémiques aux géohelminthes. De plus, nous avons mené, une étude longitudinale de 14 mois en Haiti afin d'investiguer la réponse des géohelminthes à deux traitements d'ABZ. Pour cela 344 individus vivant dans cinq communautés endémiques et naïfs pour l'ABZ, ont été enrôlés dans l'étude. Des échantillons de selles ont été collectés avant le premier traitement à l'ABZ puis deux semaines et deux mois après. Puis avant et deux semaines après le deuxième traitement. **Résultat**: Dans cette étude, des marqueurs génétiques des codons 200 chez les ankylostomes, puis 167 et 198 chez les ankylostomes, *A. lumbricoides* et *T. trichiura* ont été validés par des plasmides de contrôles et ont confirmé la capacité du test à discriminer entre des génotypes de types sensible ou résistant. Dans une étude comparative où des échantillons provenant d'Haiti, du Kenya et du Panama ont été collectés, nous avons identifié à faible fréquence avant et après traitement, le SNP 200 chez des ankylostomes du Kenya. Cependant, l'efficacité de l'ABZ était élevée. Chez *A. lumbricoides,* le SNP 167 a été identifié à forte fréquence avant et après

traitement en Haiti, au Kenya et au Panama alors que l'efficacité de l'ABZ était élevée. Chez *T. trichiura,* le SNP 200 a été détecté et il y a eu une augmentation significative de l'homozygote de type résistant, de 51.3% à 67.8% au Kenya et de 3.1% à 55.3% en Haiti. L'efficacité de l'ABZ estimée par le nombre d'œufs compté, était faible au Kenya (10.1%) et modérée en Haiti (49.7%). A partir de ces résultats, en Haiti, nous avons classés les individus infestés par *T. trichiura* en trois groupes de réponse à l'ABZ : "bonne", "intermédiaire" et "faible"réponse au traitement. Après un traitement, les fréquences des SNPs 198 et 200 ont augmenté significativement dans les groupes de réponse intermédiaire et faible. Aussi, nous avons noté une persistance du parasite et des allèles résistants deux mois et un an après traitement, chez les individus initialement classés dans les groupes de réponse intermédiaire et faible. **Conclusion** : Cette étude a montré pour la première fois, la preuve de l'existence d'une association entre les changements génétiques au niveau de la β-tubuline et la faible efficacité de l'ABZ chez *T. trichiura.*

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CONTRIBUTION OF AUTHORS

The author wrote the thesis with scientific and editorial contributions from Professor R.K. Prichard. The author under the supervision of Professor R.K Prichard performed all experiments and the majority of the epidemiological study and experimental designs presented in this thesis. Dr. R.M. Kaplan participated in the design of some experiments in manuscript 1 (Chapter 2) and in the study design and selection of endemic communities presented in the other three manuscripts (Chapter 2, 3, 4).

In the first manuscript (Chapter 2), Dr. R.M. Kaplan contributed to the design of the EHA. The optimization of the EHA on dog hookworms was conducted at the University of Georgia (GA). Dr. J.M. Schwenkenbecher provided some DNA samples of human adult hookworms used for genotyping. However, the author collected all of the human hookworm egg samples from Haiti used for the EHA and for genotyping.

In the second manuscript (Chapter 3), Dr. C.M. Halpenny and Professor M.E. Scott designed and conducted the field study in Panama and provided the eggs samples. In addition, Dr. T.S. Churcher, Professor M.-G. Basáñez, Dr. J. Kihara and Dr. C. Mwandawiro conducted and designed the field study in Kenya and provided the eggs samples. Dr. T.S. Churcher and Professor M.-G. Basáñez also provided technical advice and expertise with respect to statistical analysis. In addition, Dr. T. Streit provided the logistical coordination in the field in Haiti. Dr.

Y. Idaghdour provided guidance for some of the genetic tools used for data analysis. However, the author was responsible for the fieldwork, data collection and processing. The author also carried out genetic analysis on all samples.

In the third manuscript (Chapter 4), the author performed all of the experiments and analysis presented.

Appendix A: Dr. R. Suswillo, Dr. L. Drake and Dr. J. Kihara provided the worm samples from Kenya and conducted the field study. Dr. C. Halpenny and Professor M.E. Scott provided the egg samples from Panama and conducted the field study. Dr. J.R. Stothard provided the egg samples from Uganda and conducted the field study. Dr. D.A. Bundy provided expertise for the design of the study.

STATEMENT OF ORIGINALITY

The manuscripts contained in this thesis contributed original material and the advancement of knowledge in the field.

Chapter 2: A. Diawara, J.M. Schwenkenbecher, R.M. Kaplan and R.K. Prichard. Molecular and biological diagnostic test for monitoring benzimidazole resistance in human soil transmitted helminths. Am J Trop Med Hyg, online (doi:10.4269/ajtmh.12-048)

In this manuscript we developed sensitive diagnostic tools to monitor resistance in STHs. We validated genetic markers for polymorphisms at positions 167, 200 and 198 in the β-tubulin gene associated with resistance in *A. lumbricoides*, *T. trichiura* and hookworms. We also reported for the first time the presence of a genetic change in hookworms at position 200 in samples collected in treated areas of Haiti. In this study we also proposed novel ways of improving the *in vitro* EHA when performed on human hookworms in the field.

Chapter 3: A. Diawara, C.M. Halpenny, T.S. Churcher, C. Mwandawiro, J. Kihara, R.M. Kaplan T.G. Streit, Y. Idaghdour, M.E. Scott, M.-G. Basáñez and R.K. Prichard. Association between response to ABZ treatment and β-tubulin genotype frequencies in STHs (under revision in Plos Negl Trop Dis).

In this manuscript we applied the previously elaborated molecular diagnostic tests (for position 200, 167 and 198) to *A. lumbricoides*, *T. trichiura* and hookworm

samples collected in three different countries (Haiti, Kenya and Panama) before and after ABZ treatment. This is the first multi-site study performed to monitor ABZ resistance in STHs. In this study we report for the first time a significant increase in the resistance genotype that occurs at codon position 200 after ABZ treatment of *T. trichiura* in Haiti and Kenya and a low or moderate drug efficacy against this parasite, suggesting a possible association between the presence of the polymorphism and the poor response to treatment.

Chapter 4: A. Diawara, R.M. Kaplan and R.K. Prichard. Longitudinal studies on soil transmitted helminths in five Haitian endemic communities and association between the β-tubulin polymorphism in *T. trichiura* and response to albendazole (manuscript in preparation).

This manuscript focuses on investigating the genetic profiles of *T. trichiura* using samples collected from individual subjects showing good, intermediate or poor ABZ responses. Genetic analysis of individual and pooled eggs revealed that SNPs at codon positions 198 and 200 significantly increase after one round of ABZ treatment. It was also found that subjects showing a low and intermediate response to treatment remained infected two months and one-year post-treatment and the persistent *T. trichiura* carried a moderate frequency of the resistance allele. To our knowledge this is the first study that assesses SNP frequencies in STH samples, collected before and after ABZ treatment, in the same subjects previously naive for mass drug administration (MDA) with ABZ. In this study we also described the dynamics of infection (prevalence, parasitic intensity, and risk factors of infection) with STHs in these endemic communities. All of these

aspects made the study unique and highlight important factors necessary to maintain an effective control strategy.

Appendix A: A. Diawara, L.J. Drake, R.R. Suswillo, J. Kihara, D.A. Bundy, C. Halpenny, M.E. Scott, J.R. Stothard and R.K. Prichard (2009). Assays to detect β-tubulin codon 200 polymorphism in *Trichuris trichiura* and *Ascaris lumbricoides*. Plos Negl Trop Dis e397.

This manuscript reports the cloning of the first partial genomic DNA of the *A. lumbricoides* β-tubulin gene including the codon at position 200, which has been associated with BZ resistance in veterinary parasites. The β-tubulin sequence has been deposited in GenBank. We have also developed novel accurate DNA assays to detect SNPs at codon positions 200 in *A. lumbricoides* and *T. trichiura*. We have identified for the first time a SNP at position 200 that is associated with resistance in *T. trichiura* adults and eggs collected from untreated and treated subjects in Kenya and Panama.

LIST OF ABBREVIATIONS

ABZ: Albendazole

AH: Anthelmintic

BZ: Benzimidazole

MBZ: Mebendazole

CDC: Centers for Disease Control

CI: Confidence interval

CR: Cure rate

DALY: Disability-adjusted life years

DEC: Diethylcarbamazine

ED_{50}: Effective dose

EHA: Egg hatch assay

ELISA: Enzyme linked immunosorbent assay

ERR: Egg reduction rate

FEC: Feacal egg count

FECRT: Feacal egg count reduction test

GDP: Guanosine diphosphate

GSK: GlaxoSmithKline

GTP: Guanosine-5'-triphosphate

HW: Hookworm

Ig: Immunoglobulin

IL: Interleukin

IVM: Ivermectin

L: Larva

LD_{50}: Lethal dose

LDA: Larval development assay

LF: Lymphatic filariasis

MDA: Mass drug administration

MPA: Microtubule associated protein

NTD: Neglected tropical diseases

MT: Mutant type

PCR: Polymerase chain reaction

PSAC: Preschool-age children

RFLP: Restriction fragment length polymorphism

SAC: School-age children

SNP: Single nucleotide polymorphisms

STH: Soil-transmitted helminth

Th: T helper

TBZ: Thiabendazole

TDS: Trichuris Dysentery Syndrome

Tx: Treatment

WHO: World Health Organization

WT: Wild-type

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INTRODUCTION

The roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura* and the hookworms *Ancylostoma duodenale* and *Necator americanus* are the causative agents of geohelminthiasis. Around 24% of the global population is affected by soil transmitted helminth (STH) infections [1]. These STHs are a major public health problem in countries where they are endemic such as in Haiti, Kenya and Panama. Recognizing this, international agencies (e.g. the World Health Organization (WHO) and the World Bank), in collaboration with the national Ministries of Health in the endemic countries, have implemented control programs at the community level, based on periodic deworming with benzimidazole (BZ) anthelmintic (AH) drugs [2]. The main goals of these programs are to reduce the intensity and prevalence of infections and to disrupt the transmission of the disease. In the context of integrated programs that target different parasitic diseases, such as the program to eliminate lymphatic filariasis (LF) in Haiti, the BZ drug albendazole (ABZ) is distributed in combination with diethylcarbamazine (DEC) [3,4]. In other countries, such as Kenya [5] or Panama [6], ABZ could be selectively administered to at-risk populations such as school-age children and preschool children. All these interventions protect infected people from morbidity and allow sustainable reduction of the prevalence of infection in the entire community [2].

However, resistance to BZs, including ABZ is already established in veterinary parasites, for example in the sheep nematode *Haemonchus contortus*, and is characterized by a single nucleotide polymorphism (SNP) in the β-tubulin gene at codon 200, 167 or 198 [7-9] causing amino acid changes from phenylalanine to tyrosine (167 and 200) or from glutamate to alanine (198). These genetic changes cause a reduced or non-response of the targeted parasites to the drug. While there is still no clear evidence of resistance in human STHs, some studies have reported a reduced BZ efficacy against hookworms [10,11] and the presence of a SNP at codon 200 in whipworms [12]. The development of BZ resistance in human STHs would be a major threat to control programs in developing countries that mainly rely on BZ drugs. Thus, there is an urgent need to monitor drug resistance and AH drug efficacy in human STHs. To delay the development of a clinical problem of AH resistance, it is important to detect at an early stage SNPs associated with resistance and to establish their possible association with low or reduced BZ efficacy.

The overall objective of this thesis was to monitor in human STHs the frequency of genetic changes associated with resistance and determine their implication with regard to the development of BZ resistance. The first chapter reviews the current knowledge on STHs, their public health impact worldwide, as well as treatment strategies applied in endemic countries, the mode of action of BZ in the targeted parasite and the mechanism of BZ resistance. The second chapter presents molecular diagnostic tools developed to assess SNPs at positions 167, 198 and 200 in the β-tubulin gene of *A. lumbricoides, T. trichiura* and

hookworms, as well as the optimization of a biological egg hatch assay (EHA) to assess the response to BZ AH drugs and determine the presence of SNPs at codon 200 in hookworms collected in an area exposed to ABZ treatment in Haiti. The third chapter describes evidence of ABZ resistance-associated SNPs in *T. trichiura* from Haiti and Kenya and in hookworms from Kenya, and explores SNP frequencies in *A. lumbricoides* and hookworm samples collected before and after ABZ treatment. An association between the presence of SNPs in *T. trichiura* and the low or moderate ABZ response is also presented. The monitoring of SNP frequency is extended in the fourth chapter, which presents a longitudinal study carried out in five endemic Haitian communities. This chapter focuses on *T. trichiura* by classifying infected subjects into three groups of ABZ response; good, intermediate and poor. Results indicate an increase in SNP frequency after treatment in the intermediate and poor response groups as well as the lengthy persistence (2 months and 1 year after ABZ treatment) of the parasites carrying the resistance gene, in subjects classified at baseline as intermediate or poor responders. This chapter also describes the dynamics of infection (prevalence and intensity risk factors) of STHs in the studied area. The fifth chapter of this thesis consists of a summary and discussion of the findings presented in each manuscript and their implications for STH control programs. Finally, Appendix A describes the development of SNP markers for *A. lumbricoides* and *T. trichiura* at position 200 and presents the first evidence of a resistance-associated SNP in *T. trichiura* at codon 200. This finding initiated the longitudinal and cross-sectional investigations of SNP frequencies.

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CHAPTER I

Literature review

1.1. Public health importance of soil transmitted helminths

Soil-transmitted helminths (STHs) are a group of parasitic nematodes. There are three major nematode species of human concern because of their high prevalence and distribution worldwide, including the roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura* and two hookworms *Necator americanus* and *Ancylostoma duodenale* [1]. The global prevalence of soil-transmitted helminthiasis is estimated at more than 2 billion and remains the most common infection worldwide, of which 450 million people have significant morbidity due to their infection [2]. These infections are endemic in tropical and sub-tropical regions of the developing world with the greatest burden of infections in Sub-Saharan Africa, East Asia, China, India and South America [3] (Figure1). The high prevalence, percentage of cases and intensity of infections rank helminthiasis as one of the major neglected tropical diseases [4,5].

1.1.1. *Ascaris lumbricoides* epidemiology and clinical features of Ascariasis

A. lumbricoides is a persistent parasite that infects a quarter of the world's population and the global numbers of individuals infected is estimated to be 1.3 billion [6,7]. There are around 120-220 million new cases each year of infection with *A. lumbricoides* and school-age children (SAC) are the most at risk of infection. Among them, 59 million cases are associated with significant morbidity and about 12 million children are estimated to be at risk of acute illness, resulting in approximately 100,000 deaths per year directly attributable to complications

from infection [7]. Since infection with *A. lumbricoides* causes more disability than death and the worldwide burden is also estimated by the disability adjusted life years (DALYs), the number of healthy years lost to premature death or disability is estimated at 10.5 million years lost [8,9]. *A. lumbricoides* is the most predominant STH in the tropical and subtropical regions of the developing world [1]. The highest rate of *A. lumbricoides* occurs in China, where 47% of the population was found to be infected [10]. A high prevalence is also identified in Southeast Asia, Sub-Saharan Africa and in Central America [11] (Figure 1.1). Poverty, inadequate sanitation and climate influence the broad-scale dissemination of *A. lumbricoides*.

With respect to *A. lumbricoides*, the occurrence of the disease is related to the intensity and duration of infection and also to the host's health status [12]. Different pathologies are associated with *A. lumbricoides* according to its life cycle. During the early stage of infection, larval migration can lead to *Ascaris* pneumonitis also called Loeffler's pneumonia that is characterized by cough, asthma, fever and eosinophilia [13]. The most severe manifestations of ascariasis, experienced by children and adults, are intestinal tract obstruction caused by adult worms and the biliary complication caused by the presence of adult parasites in the biliary tree. Symptoms such as fever and acute diarrhea are associated with obstruction whereas the presence of the parasite in the biliary tree is responsible for hepatobiliary and pancreatic ascariasis [14]. It was reported that intestinal obstruction is more frequent in children less than 10 years old, explained by the

narrower intestinal lumen diameter of that age group and their vulnerability to infectivity [7].

The other global health impact associated with chronic ascariasis is the nutritional impairment caused by the damage of epithelial mucosal cells resulting in the prevention of nutrient absorption by the host that leads to stunted growth in infected children [15].

1.1.2. *Trichuris trichiura* epidemiology and clinical features of *Trichuriasis*

The global estimate of infected individuals with *T. trichiura* is around 1,049 million [16]. Among this infected population, 114 million and 223 million have been identified as being preschool-age children (PSAC) and SAC, respectively [8]. The highest intensity of infection and greatest morbidity occurs in SAC. Only in Africa, *T. trichiura* causes around 10,000 deaths annually and the global estimate of the DALYs is around 6.4 million [9].

As described for *A. lumbricoides*, the two requirements for *T. trichiura* to become a serious health problem are poor sanitation and an environment with a warm climate, high rainfall and humidity. The highest prevalence of *T. trichiura* is found in China followed by India and then Sub-Saharan Africa where 42, 36 and 27 million SAC are infected, respectively. *T. trichiura* is also present in Latin America and the Caribbean where a total of 39 million SAC are infected [11]. *T. trichiura* and *A. lumbricoides* infections are frequently concurrently present

and display a positive interaction such that infection intensity of *T. trichiura* is higher in persons with an *A. lumbricoides* infection than in those without [17].

In trichuriasis, fewer than 100 worms rarely cause clinical symptoms; however a heavier burden may result in severe complications and can sometimes lead to death. *T. trichiura* causes host injury through direct effects by invading the colonic mucosa that can cause a chronic hemorrhage resulting in anaemia [18]. Inflammation at the site of attachment results in the disruption of the normal colonic architecture and a host inflammatory response in the lamina propria [13]. The most severe manifestation of heavy infection among children is the Trichuris dysentery syndrome (TDS) which is associated with growth retardation, anaemia, dysentery and rectal prolapse [15]. Intellectual and cognitive impairments are also associated with chronic heavy infection. The long-term effects could lead to a generation of adults disadvantaged by the irreversible sequelae of infection and could compromise the economic development of their communities and nations [19].

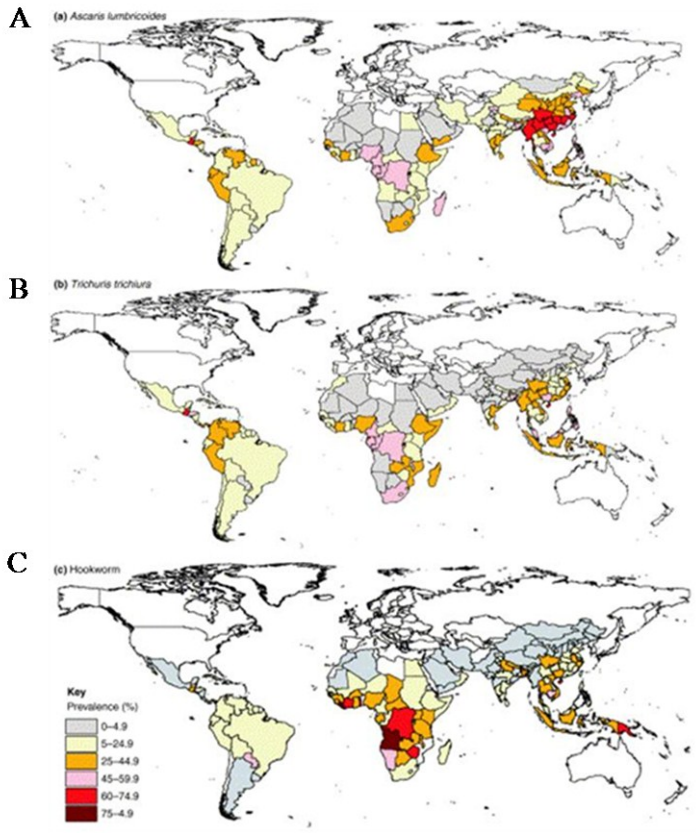
1.1.3. Hookworm epidemiology and infections

The two hookworms, *A. duodenale* and *N. americanus,* affect around 807 and 604 million people worldwide, respectively, predominantly those living in poor rural areas of the tropics [20]. Hookworm is most prevalent in eastern Asia [21] and in the coastal regions of Sub-Saharan Africa, particularly in areas with high ambient temperature such as countries near to the Sahel [22,23] (Figure 1.1). In all regions where hookworm prevalence is high, there is a close association with a low socioeconomic status [11]. Hookworms cause severe morbidity in the

host by generating intestinal hemorrhage [21] and are responsible for 65,000 deaths and 22 million DALYs lost annually [8,9].

The presence and severity of the disease depends mainly on three factors: the number of worms present [24], the species of hookworm [25], and the nutritional status of the infected host. Indeed, 25 *N. americanus* worms or less cause light infection but up to 1,000 worms can cause severe damage and symptoms in the human host. However, for *A. duodenale*, fewer worms are responsible for greater complications as seen with the *Ancylostoma* species that can suck more blood [25]. For both hookworm species, female worms ingest a minimum of 0.1 mL of blood per day [26] and patients with heavy infections may lose up to 200 mL of blood per day [27]. Also, *A. duodenale* causes more loss of pre-existing iron reserves in the host, thus children and women of reproductive age, especially pregnant women who have lower iron reserves, are particularly more at risk of developing severe manifestations of the infection [24,28]. In addition, in very heavy hookworm infections, patients may suffer from severe plasma protein deficiency leading to hypoalbuminemia, hypoproteinemia and protein malnutrition [28]. In children, chronic heavy infections are associated with growth retardation, and intellectual and cognitive impairment [24,29,30]. Finally the clinical disease is intensified by the degree of malnutrition of the host [13].

Figure 1. 1. Worldwide distribution of *Ascaris lumbricoides* (A), *Trichuris trichiura* (B) and Hookworms (C).



Source: [11]

1.2. Soil-transmitted helminths and their life cycles

The life cycle of the three species of STHs does not involved an intermediate host; humans are the definitive host.

1.2.1. Morphology and life cycle of *A. lumbricoides*

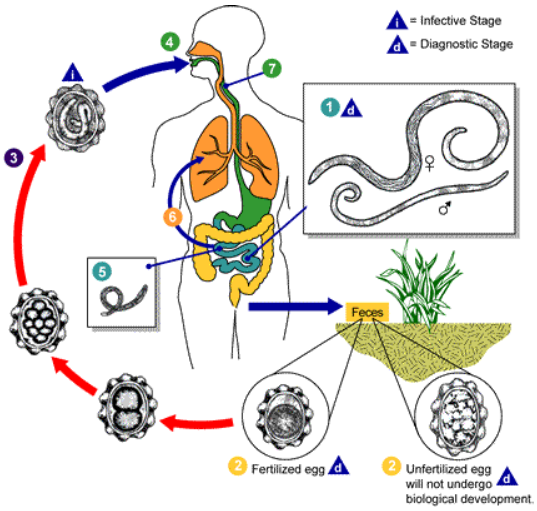
Classification: Nematoda; Rhabiditea; Ascaridida, Ascarididae, *Ascaris*

A. lumbricoides is one of the largest nematodes. The morphological characteristics are summarized in Table 1. The male worm measures 15 to 31 cm long by 2 to 4 mm wide and its posterior end is curved ventrally. Females are 20 to 49 cm long and 3 to 6 mm wide. The ovaries are extensible and the uteri may contain up to 27 million eggs at a time with 200,000 being laid per day [31]. Adult worms have an average longevity of 1-2 years and female worms can release eggs for one year and sometimes for a period of 20 months [32]. Fertilized and unfertilized eggs are passed in the feaces in the unembryonated state and are extremely resistant to low temperature, desiccation and strong chemicals. However embryogenesis is delayed by such factors [13]. Under advantageous conditions of a warm climate, humidity, moisture-retaining soil and dense shade, within 9 to 13 days fertilized eggs moult to the first and second stage (infective stage) of embryonation. Fully embryonated eggs, containing the infective larvae L3 can survive in the environment for up to 15 years [33].

Humans become infected with *A. lumbricoides* by accidental ingestion of embryonated eggs, which hatch in the duodenum and from there the larvae migrate to the liver, pass through the right side of the heart and enter into the pulmonary circulation. While in the lungs, the fourth moult occurs and takes approximately 10 days for L3 to develop to the L4 stage and grow to a length of 1.4 to 1.8 mm. They then move up to the pharynx where they are swallowed and enter the small intestine where they mature into adult worms. Many of the larvae, however, do not complete the migration and end up accumulating in almost every organ of the body causing acute tissue reactions [34]. The cycle from ingestion of

eggs to the production of fertilized eggs by female *A. lumbricoides* takes 2 to 3 months [34]. The life cycle of *A. lumbricoides* is illustrated in Figure 1.2.

Figure 1. 2. Life cycle of *Ascaris lumbricoides*



Source : http://www.dpd.cdc.gov/dpdx/HTML/Ascariasis.htm

1.2.2. Morphology and life cycle of *T. trichiura*

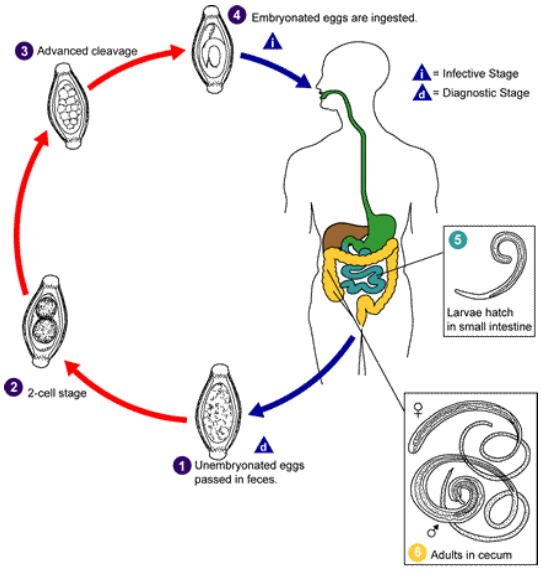
Classification: Nematoda; Adenophorea; Trichurida, Trichuridae, *Trichuris*

T. trichiura, the human whipworm is a much smaller worm than *A. lumbricoides*. Adult worms measure 30 to 50 mm long with females being larger than males [35]. Both sexes have a single gonad and the anus is near the tip of the tail. In females the vulva is near the junction of the oesophagus and the intestine. The uterus contains many unembryonated barrel-shaped eggs that measure 50-54 µm in length and 22 µm in width [15]. Each female worm produces from 3,000 to 20,000 eggs per day [35]. The eggs require a period of approximately 21 days in

the soil, which must be moist, warm, and shaded, to complete embryonation and generate the infective larvae (L1) [13].

Infection with *T. trichiura* occurs by ingestion of infective eggs that hatch in the duodenum where the L1 grow for approximately one week and then re-enter the intestinal lumen and migrate to the caecum where they burrow into the mucosal surface by means of their anterior ends. Larvae pass through four moults before maturing into adult worms. *T. trichiura* adults take 1 to 3 months to develop following infection, and after mating the females start to lay eggs. Adults live for several years in the colon [13] (Figure 1.3).

Figure 1. 3. Life cycle of *Trichuris trichiura*



Source : http ://www.dpd.cdc.gov/dpdx/html/trichuriasis.htm

1.2.3. Morphology and life cycle of hookworms: *A. duodenale* and *N. americanus*

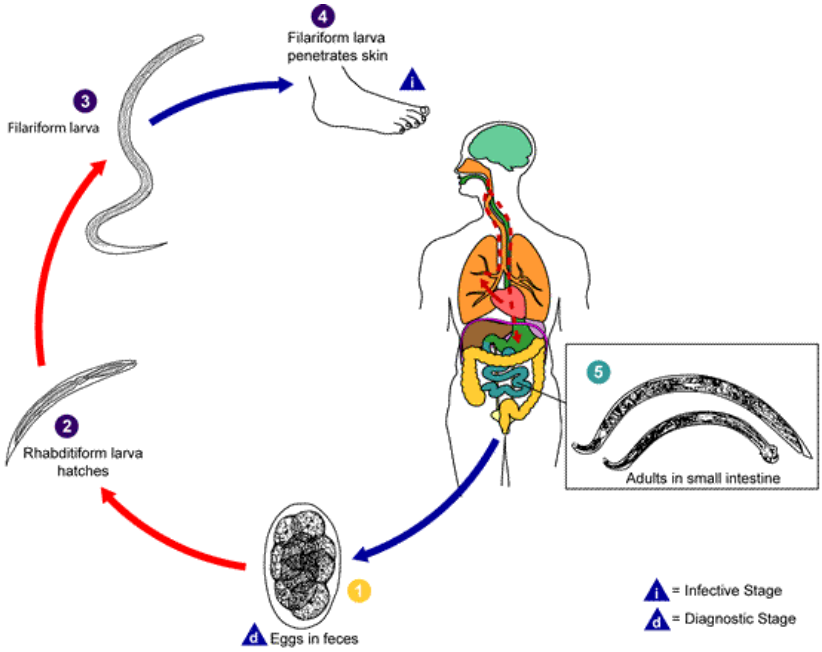
Classification: Nematoda; Rhabditea; Strongylida, Ancylostomatidae, *Ancylostoma duodenale* and *Necator americanus*

The two major hookworm species, *A. duodenale* and *N. americanus,* have similar morphologies. The anterior end of adult worms is curved dorsally giving the worm a hook-like appearance. In females, the vulva is post-equatorial and two ovaries are present. Female worms produce several thousand eggs per day for up to nine years. Adult *N. americanus* males are 7 to 9 mm long while females are 9 to 11 mm long. They produce about 5,000 to 10,000 eggs per day and the normal life span is 3 to 5 years [36]. Adult *A. duodenale* males measures 8 to 11 mm long and female worms are 10 to 13 mm long with the vulva located about a third of the body length from the posterior end. A single female can lay from 10,000 to 30,000 eggs per day and the normal life span is one year [36].

Once released in the feaces, unembryonated eggs require adequate warmth, shade and a moist environment for continued development and subsequently hatch to the free-living L1 stage within 1 to 2 days. The newly hatched worms live in the feaces feeding on faecal matter and bacteria, and moult to the second L2 stage in 2 to 3 days. The L2 continue to grow and feed, and after about 5 days moult to the third L3 stage which is the infective stage. L3 hookworms live in the upper millimetres of the soil and are very sensitive to freezing and desiccation. They migrate up to the surface of the ground when the soil is wet and extend themselves like snakes to maximise their opportunity for

contact with a host. They can live in a free-living infective stage for several weeks under ideal conditions. Infection occurs when the L3 contacts the skin and burrows into it. *N. americanus* must penetrate the skin to infect humans while *A. duodenale* can also penetrate oral mucosa and be passed in mother's milk and probably be acquired transplacentally [37,38]. After gaining entry into a blood or lymph vessel, L3 hookworms migrate to the heart, the lungs and the trachea where they are swallowed and finally arrive in the small intestine. There, they attach to the mucosa, grow and moult twice before maturing into adult worms. Five weeks are required from the time of infection to the beginning of egg production. However, *A. duodenale* can undergo developmental arrest for up to 38 weeks, with their maturation coinciding with the seasonal return of environmental conditions favourable for transmission [39]. The life cycle of hookworms is displayed in Figure 1.4.

Figure 1. 4. Life cycle of hookworms



Source : http://www.dpd.cdc.gov/dpdx/html/Hookworm.htm

In, Table 1.1, the characteristics of the life cycle of *A. lumbricoides*, *T. trichiura* and hookworms (*A. duodenale* and *N. americanus*) are summarized.

Table 1. 1. Specific aspects of the life cycle of *Ascaris, Trichuris* and hookworms. [1]Hookworm. [2]Time to reach the infective stage.[3]Time from egg ingestion to egg production.

Characteristics	*A. lumbricoides*	*T. trichiura*	*N. americanus*[1]	*A. duodenale*[1]
Infective stage	Embryonated eggs with L3 larvae	Embryonated eggs with L1 larvae	L3 larvae	L3 larvae
Time for embryogenesis[2]	9 -13 days	21 days	7-8 days	7-8 days
Egg production per day	200,000	3,000-200,000	5,000-10,000	10,000-30,000
Adult life span (years)	1-2	1-2	3-5	1
Duration of the life cycle[3]	2-3 months	1-3 months	5 weeks	5 weeks

1.3. Immunology of soil-transmitted helminth infections

As mentioned in the previous section, STH adult worms have the ability to survive for many years within the host where they manipulate its immune system by establishing a chronic infection and by evading host defences [20]. Thus, STHs are thought to persist within the human host not only by evading the host immune system but also by creating niches that optimize successful feeding and reproduction [40].

1.3.1. Immunity and soil-transmitted helminths

STHs induce a humoral response characterized by the release of T-helper 2 (Th2) cytokines such as interleukins including IL-4, IL-5, IL-6, IL-10, IL-13 as well as the mobilization of specific effector cells such as mast cells, eosinophils and basophils, as well as immunoglobulin E (IgE) [40,41]. Thus, Th2 cytokines and effectors induce a decrement in egg production and contribute to worm expulsion [42,43]. Their mode of action varies according to the nematode species; for instance, mast cells seem to be a central element involved in the protective response against *Ascaris* and hookworm species but are not important for the expulsion of *Trichuris* [44,45]. Regarding hookworms, it has been described that after anthelmintic treatment, IL-5 protects against the reinfection of *N. americanus* by acting against incoming larvae [46]. In addition, in *Ascaris* and *Trichuris*, it has also been shown that IL-5 was positively associated with the prevention of reinfection. Continuing exposure to STHs allows the host to sustain protective immune responses as a means to control the infection [42,43].

1.3.2. Evasion of immunity

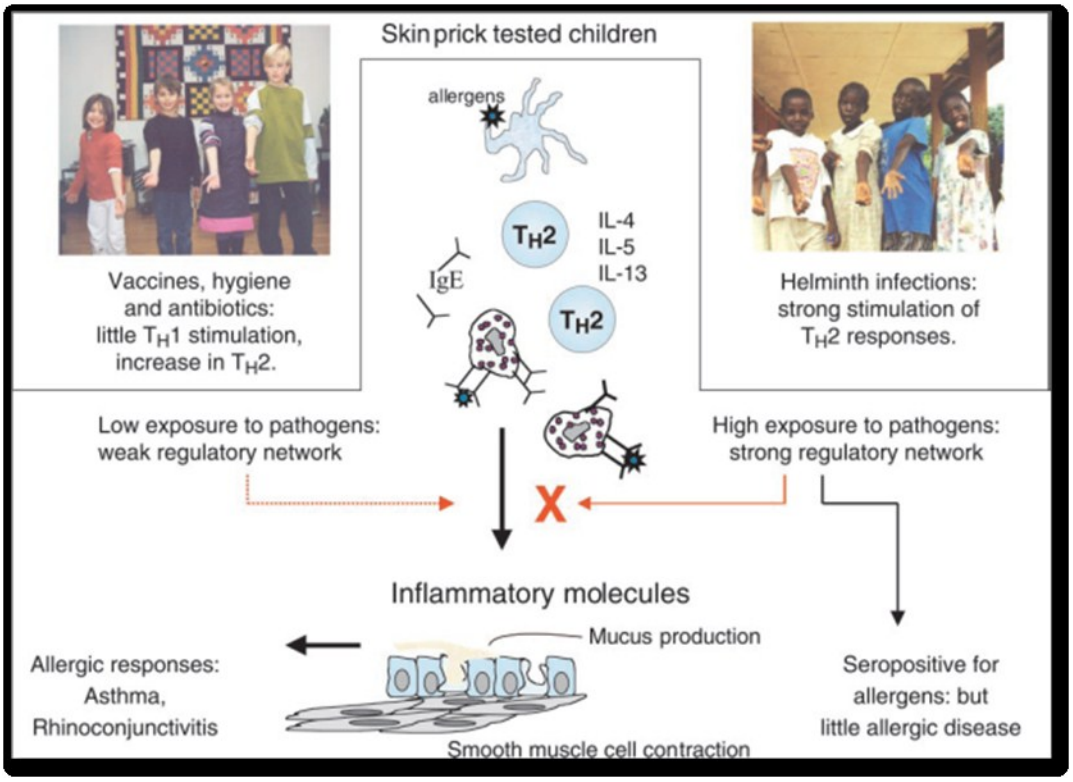
It has been shown that these gastrointestinal nematodes could evade the host immune system by using several mechanisms [47]. *In vitro* tests on *N. americanus* have demonstrated that the exterior cuticle plays a crucial role in the survival of larvae within the host by shedding cuticle antigens able to cleave the Fc portion of immunoglobulin M (IgM), IgG and IgA [48,49]. Hookworms are also capable of producing molecules such as metallopeptidases that confer a defence against host immune attacks. Another important feature is the suppressive effect that nematodes have on the host immune responses [50]. For instance, *Ascaris suum*, the species of *Ascaris* found in pigs, releases immunosuppressive molecules that interfere with antigen presentation [51]. Besides immunological features, host-specific and environmental factors have been identified that can influence the risk of acquiring or harboring heavy-intensity helminth infections.

1.3.3. Allergy and helminths

Many studies have investigated the association between allergy and helminth infections and found that the Th2 environment induced by STH infections shared characteristics with the allergic response [52]. Indeed, allergic diseases including asthma and other atopic diseases are an important cause of morbidity in developed countries and in urbanized areas of the developing world, causing chronic disease in childhood [52-54]. However, their occurrence is still low in rural areas of developing countries [55-57]. It has been proposed that in the developed world, the low exposure to pathogens caused by the high prevalence of vaccination, the regular administration of antibiotics and good hygienic conditions could

negatively alter the immune system response. Thus, the immune system receives insufficient Th1 stimulation (induced by microbes) and consequently, Th2-associated pathologies such as asthma become more predominant. This theory is the so-called the "hygiene hypothesis" [58] (Figure 1.5). Further studies on animal models have shown the potential implication of helminths on the hygiene hypothesis. In animal models, it has been shown that parasitic helminths could act as modulators of the allergic response, either by enhancing allergic inflammation or by suppressing it [59,60]. The immune mechanism that links helminths and allergy involve blocking of IgE in the case of a negative association. Helminth infections are characterized by the release of highly polyclonal IgE that saturates mast cell receptors (FcεRI) preventing the binding of allergen-specific IgE thereby inhibiting degranulation [60]. A study conducted in Venezuela on the association between helminths and allergy in individual subjects, revealed that heavy burden helminth infections conferred protection to allergy compare to those harbouring a light burden of helminths [61]. Drug treatment reduced the clinical allergic symptoms in individual subjects with a light infection [62] and inversely exacerbated the symptoms in those who had a heavy parasitic burden [63].

Figure 1. 5. The Hygiene Hypothesis



Source [60]

1.4. Control of Soil-transmitted helminths

1.4.1. Anthelmintic drugs for soil-transmitted helminths

The current strategies of STH infection control programs are based on (i) large scale distribution of anthelmintics (AHs) to reduce morbidity by decreasing the worm burden, (ii) development of appropriate sanitation to control transmission by decreasing water and soil contamination, and (iii) health education promoting hygiene to reduce transmission and reinfection [9]. The WHO recommends four front line drugs for the treatment of STH infections:

albendazole (ABZ), 400 mg tablets given as a single dose and reduced to 200 mg for children less than 2 years old; mebendazole (MBZ), 500 mg administered as a single dose; levamisole (40 mg) and pyrantel (250 mg) tablets given as a single dose by weight (2.5 mg/kg and 10 mg/kg for levamisole and pyrantel pamoate, respectively) [9]. However, ABZ and MBZ that belong to the benzimidazole (BZ) class of AHs are the most commonly prescribed drugs since dosage does not vary according to weight so they are easier to administer to communities [64].

1.4.2. Indicators of drug efficacy

AH efficacy is determined by two indicators, the cure rate (CR), a qualitative measure of the infection after treatment (presence or absence of eggs in stool) and the faecal egg reduction test (FECRT), a quantitative measure based on the egg reduction rate (ERR): the percentage reduction in eggs per gram of feaces before and after treatment [65]. Many studies on STHs have investigated the CR and ERR to determine drug efficacy. However, there is a large discrepancy between studies [66] due to the lack of standardization of these indicators and confounding associated factors [67].

First of all, in many studies only the CR is assessed. However, this indicator has some major limitations. It is mainly influenced by the baseline intensity [66] and so in highly endemic communities with a high STH intensity [68], the CR will be lower than in ones where the baseline faecal egg count (FEC) is lower [69]. In a recent study, it has been reported that for high pre-treatment FEC, the CR was not reduced in *Ascaris*, *Trichuris* and hookworms [70]. Futhermore, the CR highly depends on the sensitivity of the diagnostic test used

to determine FEC, thus for a low sensitivity test such as Kato-Katz, the number of false negatives will increase and so the CR will appear better than with a more sensitive parasitological method [71]. For these reasons, the CR is thought to be an inappropriate indicator for assessing drug efficacy against STHs in mass drug programs [70,72]. In veterinary parasitology, the procedure to determine drug efficacy has been standardized and the FECRT is recommended [73]. This test also seems to be more appropriate for monitoring drug efficacy against STHs in public health [70].

Guidelines on the use of the FECRT have been provided by the WHO. In this report thresholds for drug efficacy are defined for the three STHs and recommendations on stool sample procedures and sample size are also provided [74]. Despite this, the FECRT has also been criticized due to confounding associated variables that directly affect accurate monitoring of drug efficacy. By definition, the FECRT measures the change in faecal egg output in individual subjects before and after drug treatment. However, in hookworms the egg output is density dependent meaning that there is an inverse correlation between the burden of worms and the eggs released by the females [75]. This particular feature may lead to a strong bias as the variation from day to day in STH egg excretion has been described in previous studies [76,77]. In addition, as mentioned above, the FECRT is also mainly influenced by the sensitivity of the parasitological test employed [71].

1.4.3. Benzimidazole efficacy against STHs

A meta-analysis reported that a single dose of 400 mg of ABZ or 500 mg of MBZ is highly effective against *A. lumbricoides* and resulted in CRs of 88% (95% CI, 79%-93%) and 95% (95% CI, 91%-71%), respectively. More variable efficacy was reported against *T. trichiura* with CRs of 28% (95% CI, 13%-39%) and 36% (95% CI, 91%-97%) for ABZ and MBZ, respectively, and against hookworms with CRs of 72% (95% CI, 59%-81%) and 15% (95% CI, 1%-27%) for ABZ and MBZ, respectively [66]. In many cases, a single dose of MBZ shows a low CR against hookworms whereas ABZ achieves a satisfactory CR [78,79]. Neither a single dose of ABZ nor MBZ is effective against *T. trichiura*, although, multiple dosing increases efficacy [80,81]. Thus, in areas of intensive transmission, treatment two or three times a year is necessary to reduce morbidity whereas in areas with lower intensity of transmission, a yearly treatment is sufficient [82,83]. The low efficacy of BZ against *T. trichiura* and hookworms is a major concern for STH control programs that mainly rely on drug treatment. There is, therefore, an urgent need for drug discovery and development of new drugs.

1.4.4. Drugs in development for STHs

The antiprotozoal drug nitazoxanide patented as a cestocide has been reported to have properties against gastrointestinal parasites [84]. Multiple doses of nitazoxanide (200 or 500 mg) have demonstrated high CRs against *A. lumbricoides*, *T. trichiura* and hookworms [85-87], thus nitazoxanide has been proposed as a potential drug candidate for human soil-transmitted helminthiasis

[88,89]. However, a recent study has reported that a single high dose of nitazoxanide (1,000 mg) showed poor efficacy against *T. trichiura* resulting in a CR of only 6.6% (95% CI, 2.4%-10.8%) and an ERR of 13.4% (95%CI, 0.0-33.7%) [90]. To date, tribendimidine is the only AH for STHs in its late stage of development [91]. This drug has been developed and approved in China for use in humans [92]. A single dose of tribendimidine (200 or 400 mg) was reported to be safe but showed no or low CRs against *T. trichiura* [91,93]. In spite of the effort made to develop and find new candidates against STH infections, there is still an urgent need to develop a compound with high efficacy against *T. trichiura* [90,94]. However, a recent study gives new hope by presenting another potential drug candidate against *Trichuris*. *In vitro* and *in vivo* experiments with the murine species, *Trichuris muris* releaved the high trichuricidal activity of oxantel pamoate. Further studies are necessary to confirm the activity of oxantel pamoate against the human whipworm *T. trichiura* [95].

1.4.5. Control program through mass drug administration

The mass drug administration (MDA) of AHs at the community-level is the main strategy adopted by control programs to sustainably reduce the morbidity, prevalence and transmission of STHs in the community [96]. The expectations of community-based MDA programs are to reduce the prevalence of infection to a level at which it ceases to be of public health significance [97]. Community-based interventions do not specifically target a specific group for treatment but rather the entire community is subjected to treatment [96], such as the onchocerciasis control program that has been implemented as a community-

directed treatment initiative with ivermectin (IVM) as well as the lymphatic filariasis (LF) elimination program where diethylcarbamazine (DEC) or IVM is given in combination with ABZ [98,99]. Thus, the major drugs employed in the LF and onchocerciasis control programs also target the STHs and thereby provide health benefits to the community by reducing infection with STHs [100,101].

In other cases, MDA programs could be targeted to the groups who are at most risk of STH infection such as SAC. Targeting AHs to SAC through school-based health programs is aimed at deworming children of STHs. Delivery of deworming pills to schools can be achieved at low cost with the help and support of school staff and parents [12,102]. The administration of these agents reduces egg counts by more than 70% [103] and worm burden to a level below the threshold that could result in disease [104]. Regular deworming contributes to improved health and nutritional status for SAC and in turn leads to an increase in school attendance and educational achievement [19,99]. On the other hand, children are not the only group at risk who could benefit from AH treatment. When combined with iron and folate supplements, these drugs control maternal anemia during pregnancies [105].

Regular treatment with front-line AHs has proven their positive effects, so treatment programs will be maintained in future years to control morbidity due to helminth infections in endemic countries [106].

1.5. Diagnosis of soil-transmitted helminth infections

Diagnosis of STHs relies on the microscopic detection of eggs in faecal samples. Therefore, various parasitological techniques have been employed in order to identify STH eggs and, to date, use of the Kato Katz thick smear method is recommended by the WHO for detection and quantification of STH eggs in human faecal material [74]. This method is widely used because of its simplicity, minimal equipment requirement, low cost [107] and suitability for use in the field. However, a single stool sample examination has a low sensitivity for quantifying eggs, in particular for light STH intensity infections an underestimation of the number of eggs is common [108]. The sensitivity is also decreased for polyparasitism infections as helminth eggs appear at different clearing times [109]. For instance, some studies have found that hookworm eggs disappear in the cleared slides if the time of examination exceeds 30 min, resulting in a false negative. To increase the sensitivity of Kato-Katz, some studies have suggested performing multiple examinations [110,111]. All of these factors point to the need for the standardization of this method particularly when applying it in large-scale studies [112,113]. Several alternative diagnostic tests such as McMaster and FLOTAC have also been proposed for monitoring drug efficacy.

1.5.1. Comparison between Kato-Katz and McMaster tests

McMaster is a quantitative flotation technique mainly used for veterinary nematodes [114] and has been evaluated for use in large-scale studies on human STHs [70,115,116]. A qualitative comparison showed that Kato-Katz had a higher sensitivity for detection of STHs [115,116]. This difference is partially explained

by the particulars of the procedure [107]. Indeed more stool material is used when employing Kato-Katz than McMaster (41.7 mg versus 20 mg, respectively) [115]. Quantitative evaluation revealed a higher FEC for *A. lumbricoides* [115,116] and hookworms [116] with Kato-Katz, compared to McMaster, but no quantitative difference between techniques was detected for *T. trichiura* [115,116].

Overall, both tests are simple and feasible to be performed under field conditions [115,116]. McMaster does not necessarily require a centrifuge [117]. Comparative studies between Kato-Katz and McMaster have suggested that the latter is a promising technique for monitoring drug efficacy in STHs because of its ability to detect multiple infections without any time constraints (in contrast to Kato-Katz) [115-117] and its robust multiplication factor [115].

1.5.2. Comparison between FLOTAC and Kato Katz

FLOTAC is also a quantitative technique used in veterinary and human parasitology [118] and has demonstrated more sensitivity than the other tests. It has been reported that a single FLOTAC is more sensitive than multiple Kato-Katz for detecting low-intensity STH infections [110,119,120]. This flotation method improves the ability to detect human hookworms in contrast to Kato-Katz [120,121]. However, some disadvantages of the FLOTAC technique have been highlighted and include higher cost, longer time required to train laboratory personnel, and longer time to prepare and examine stool samples [122,123]. In addition, FLOTAC still needs to be validated for use in large-scale studies for monitoring drug efficacy against human parasites [71,116].

Thus, the "gold standard" test to diagnose STHs does not exist but several techniques are already available, and among them, the FLOTAC method holds the most promise as the tool to be used for STH monitoring and surveillance in helminth control programs [119,123].

Immunodiagnostic techniques such as the enzyme-linked immunosorbent assay (ELISA) can also be used to diagnose STH infections [124]. However, its use is not feasible in field laboratories with inadequate resources, as serological tests require blood collection and expensive reagents and infrastructures. Therefore, currently, this technique can not be routinely used as a diagnostic tool in endemic countries [125].

1.6. Benzimidazoles

BZ were initially developed as plant fungicides [81] and later for veterinary and human medicine and show activity against cestode, trematode and nematode parasites [126,127]. The variation in efficacy between different BZ drugs could be partially explained by their pharmacokinetic properties [128].

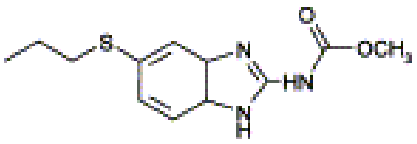
1.6.1. General description and pharmacokinetics of albendazole and mebendazole

ABZ (5-(propylthio)-1*H*-bezimidazol-2-yl) is a carbamic acid methyl ester (see Figure 1.6) and has vermicidal, larvicidal and ovicidal activity. In 1982, ABZ, synthesized by SmithKline Beecham Pharmaceuticals, was approved for the treatment of human intestinal helminthiasis [103]. In adult patients it is

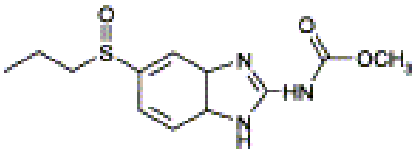
administered as a single oral dose of 400 mg and has few side effects attributed to it [81] as attested by a study that showed no significant differences in terms of side effects between the placebo and treated groups [129].

ABZ is poorly soluble in water as well as in many organic solvents and consequently is hardly absorbed from the gastrointestinal tract [128,130]. However, ABZ is rapidly metabolized in the liver into a more soluble sulphoxide derivative (albendazole sulphoxide) and further oxidation results in formation of the sulfone (albendazole sulfone) (Figure 1.6). Thus, the concentration of ABZ in the plasma is either very low or undetectable [130]. It has been reported that the oral availability of ABZ and the sulphoxide could be enhanced by a fatty meal or just eating and could increase absorption by up to 5-fold [131,132]. ABZ sulphoxide is widely distributed throughout the body but it is mainly located in the plasma with a half life between 8-12h in man [133]. Excretion of ABZ metabolites mainly occurs in the urine but also in the bile [130,133].

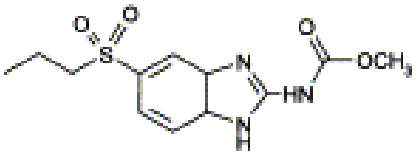
Figure 1. 6. Albendazole, albendazole sulphoxide and albendazole sulfone



Albendazole (ABZ)



Albendazole sulphoxide (ASOX)



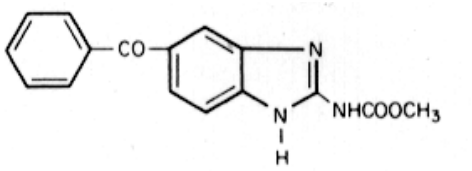
Albendazole sulphone (ASON)

Source [128]

MBZ is methyl 5-benzoyl-1*H*-benzimidazole-2-yl-carbamate (Figure 1.7). It was introduced in 1972 by Janssen Pharmaceuticals for use in humans against intestinal helminths and since 1980 it has been widely used in mass treatment programs [103]. MBZ is administered as a single oral dose of 500 mg in man [9]. As a result of its low solubility in water and organic solvents, this drug is poorly absorbed by the gastrointestinal tract. Consequently, MBZ has a low bioavailability. Like absorption of ABZ when given orally in man and animals, [128,134] the absorption of MBZ could be enhanced when the dose is taken with a fatty meal. The metabolism of MBZ involves ketoreduction, carbamate

hydrolysis and conjugation [134] whereby MBZ is metabolized into hydroxyl (OH-MBZ) and amino (NH2-MBZ) derivatives. These conjugates are not biologically active and contrary to ABZ metabolites, their contribution to anthelmintic efficacy is very low [127,135]. The most important excretory pathway in man for the metabolites and the parent drug is the bile, urine and feaces, however, an additional pathway through milk could also be involved [128].

Figure 1. 7. Mebendazole



Source:

http://www.science.smith.edu/departments/Biology/SWILLIAM/fgn/pnb/treat.html

1.6.2. Mode of action of benzimidazoles

Once BZs, taken orally, enter the human host they subsequently reach the specific receptor of the helminth parasites, found in the host's blood or intestinal contents, by diffusion through the external surface of the parasite [136]. However, it is thought that passive drug transfer by transcuticular transport is the most important route for drug diffusion [136,137]. *Ex vivo* experience with ABZ uptake in the roundworm *A. suum* highlights the critical role of the cuticle for drug absorption in nematodes as the diffusion through the cuticle mainly depends on the lipophilicity of the drug [137,138]. Thus, this is a major determinant as it

allows the drug to be delivered at an effective concentration to the targeted receptor within parasitic cells where the drug exerts its therapeutic effect [136,138].

The most important mechanism of action exerted by BZs remains their binding to the parasites tubulin and, thus, blocking microtubule matrix formation [139]. Indeed, this inhibitory effect was first demonstrated by studies in fungi [140]. It was subsequently confirmed in nematodes by showing that MBZ interferes selectively with microtubules of *A. suum* while the host's microtubules remain unaffected [141]. Subsequent studies on receptor binding affinity confirmed that the anthelmintic MBZ interferes and disrupts cytoplasmic microtubules of parasitic nematodes by binding to the β-tubulin subunit [142,143]. In addition it was found that the efficacy of BZs correlates with their binding affinities for tubulin [143]. In the sheep parasite *Haemonchus contortus*, BZs exert their effect in the nematode's intestine by disrupting microtubules [144]. Many studies have investigated the specific tubulin binding site of BZ, and one study in particular, based on the use of a photoactive fenbendazole derivative, identified the binding domain of BZs on β-tubulin isotype 1 as being between amino acids 63 and 105 [145]. Later, the BZ binding site was proposed to include α and β-tubulins and the planar BZ ring that binds between the phenylalanines at positions 167 and 200 [146]. However, it has been suggested that this proposed model for the BZ binding site is in contradiction with the tubulin crystal structure [147,148].

1.6.3. β-tubulin and microtubules

Tubulin heterodimers, made up of α and β tubulin, are the basic structural components that constitute microtubules. These subunits have a molecular weight of approximately 50 KDa and have over 40% similarity in their nucleotide sequence. [139]. The heterodimer associates in a head-to-tail polar fashion to form linear protofilaments and then associates through side-to-side interactions to form a microtubule [149]. Thus, microtubules are polar and the positively charged end where the tubulin dimers bind is more dynamic than the negatively charged end. Microtubule assembly and disassembly requires the binding, exchange and hydrolysis of guanosine-5'-triphospohate (GTP). The GTP bound to α-tubulin appears to play a structural role and cannot be exchanged whereas the GTP on β-tubulin can be exchanged with GTP from the solution. The β-tubulin associated GTP is hydrolyzed to guanosine diphosphate (GDP) that provides the driving force for microtubule dynamics [150]. This dynamic equilibrium between microtubules and the heterodimer alternates between periods of growing and shrinking through the addition or removal of tubulin subunits at the end of microtubules [151]. The equilibrium may be disturbed by changes in temperature or by endogenous co-factors, such as GTP, Mg^{2+}, Ca^{2+} and microtubule associated proteins (MAPs) [139]. Microtubules are filamentous and hollow cylindrical structures with an approximate lumen diameter of 15 nm and an outer diameter of 25 nm [139]. They are involved in many aspects of the cell structure, motility and intracellular transport [152].

Genetic and biochemical evidence suggests a critical role for β-tubulin in BZ activity [153]. By binding to microtubules, BZs prevent the self-association of subunits onto the growing microtubules and disrupt the equilibrium by inhibiting the polymerization of tubulin. This results in capping of the microtubule at the associating end while the microtubule continues to dissociate from the opposite end, with a net loss of microtubule length [154].

Beta-tubulin isotypes are highly conserved in many vertebrates [155]. In the human hookworms, *N. americanus* and *A. duodenale*, the β-tubulin isotype 1 gene has been completely sequenced (GenBank accession number: EF392851 and DQ055415, respectively). The genomic sequences are highly conserved among the hookworm species with lengths of 3,889 bp for *A. duodenale* and 3,106 bp for *N. americanus*. β-tubulin has also been cloned from the zoonotic hookworm *Ancylostoma caninum* [156]. In *A. lumbricoides* the β-tubulin isotype 1 gene has been partially sequenced (GenBank accession number: FJ501301.1) [157]. The *T. trichiura* β-tubulin cDNA clone (Genbank accession number: AF118385) was sequenced and revealed a coding sequence of 1,332 bp encoding a protein of 444 amino acids that showed 90% identity with β-tubulin of *Onchocerca gibsoni Tbb, Brugia pahangi* β1*, H. contortus gru-1* and *Caenorhabditis elegans ben-1*. Southern blot analysis demonstrated that *T. trichiura* had a single β-tubulin isotype present at a single locus [158]. The same genomic characteristic was observed in filarial nematodes with the exception of *B. pahangi*, which possesses two β-tubulin isotype genes [159]. In *H. contortus,* three β-tubulin cDNA sequences, β8-9, β12-16, and β12-164 have been isolated [160]. It was later

confirmed that β12-16 and β12-164 belonged to the same isotype [161]. Recently two additional β-tubulin isotypes have been identified in *H. contortus* [162].

1.6.4. Benzimidazole resistance

Resistance to the BZ class of anthelmintics was reported in the veterinary nematode *H. contortu*s in the late 1970s. The resistance identified was against thiabendazole and appeared to be inherited as an autosomal feature [163]. Studies involving the non-parasitic nematode, *C. elegans,* showed that the single locus, BEN-1, encoding a β-tubulin appeared to be the only BZ-sensitive β-tubulin in this nematode. Interestingly, animals lacking BEN-1 were viable in the presence of BZs. This supported the hypothesis that β-tubulin isotypes might be homologous in function [164]. Further investigation on the characteristics of BZ resistance revealed a reduced affinity of tubulin for BZs in resistant isolates, such as in the nematodes *Trichostrongylus colubriformis* of herbivorous mammals [165]. This reduced affinity was also dependent on the structure of BZ [166]. Similarly, in *H. contortus* the resistance was also associated with a reduction in the stability of the BZ-tubulin complex and this change in binding correlated with a loss of high-affinity receptors [143,167]. Further elaboration identified a clear association between a single nucleotide polymorphism (SNP) conferring an amino acid substitution of phenylalanine for tyrosine in the β-tubulin gene at codon position 200 and the resistant phenotype of the parasite [168]. In the same species and in *T. colubriformis* it was found that BZ resistant populations showed a decrease in β-tubulin polymorphism as the result of selection that was considered the major means of acquiring BZ resistance [169-172]. Analysis of *H. contortus*

β-tubulin isotype 1 DNA sequence confirmed that the identified amino acid substitution mediated BZ resistance and showed a correlation between the reduction in polymorphism and the increase in resistance [173]. This β-tubulin isotype 1 gene polymorphism has frequently been considered as the most important mutation conferring resistance to BZ [168]. However, resistance could also be enhanced by a deletion of the β-tubulin isotype 2 gene in highly resistant strains [161]. Similarly, a study found that in *H. contortus* both β-tubulin loci were involved in BZ resistance and it was hypothesized that selection was due to changes in allele frequencies rather than to novel genetic rearrangements. This suggested that resistance alleles were present (at low levels) in the population prior to the occurrence of selection [170]. The mutation at position 200 in β-tubulin isotype 1 appears to be recessive and the most common in parasitic nematodes [174,175].

Resistance to BZs was also associated with other codons in β-tubulin genes. Investigations into the distribution of codon 200 in equine C*yathostomum* populations, suggested that this mutation was not the only one responsible for resistance, and that a phenylalanine to tyrosine mutation at position 167 of β-tubulin isotype 1 was also associated with resistance [176-178]. Even if both substitutions at codons 200 and 167 have been observed, they seemed not to occur concomitantly, such that the homozygote resistant at one codon was found in combination with the homozygote susceptible at the other codon [176]. Thus, double mutants were never identified in either cyathostomins or trichostrongyles, suggesting that mutations at both loci could be lethal [174,176,179], or that both

mutations occurring at the same time is exceedingly rare [180]. In addition, an alternative point mutation, the SNP at codon position 198 conferring the amino acid substitution of glutamate for alanine was identified as being involved in the mechanism of resistance [181,182]. Even if this substitution at codon position 198 is less prevalent than the one at codon position 200, *in vitro* experiments that select for BZ resistance in *H. contortus*, confirmed the association of this SNP with the resistance phenotype of the worm [138,183].

The possible reversible feature of resistance was investigated and it was found that there was no significant difference between the relative fitness of BZ-resistant and sensitive strains of *T. circumcincta* [184]. Another report demonstrated that despite the absence of BZ treatment and the administration of levamisole for many years, resistant strains of *H. contortus* did not revert to susceptible ones [185]. Together these results suggest that BZ-resistance is irreversible.

Therefore, evidence clearly demonstrates that polymorphisms in β-tubulin genes are responsible for BZ resistance through prevention of drug binding [146,186].

1.7. Anthelmintic resistance in human soil-transmitted parasites

In veterinary parasites, widespread and frequent use of BZ drugs has led to extremely high rates of resistance in multiple nematode species in a wide range of hosts. For human parasites the same class of AH drugs are massively used in

endemic countries. Thus, there is a concern that AH resistance could develop in human STHs.

Some studies have reported poor drug efficacy or failure of a drug treatment against hookworms and, therefore, strongly point to the development of drug resistance. For instance, in Mali the failure of MBZ to treat *N. americanus* has been described [187]. However, a following study carried out at the same sites failed to report evidence of drug resistance with MBZ [68]. Another study conducted in Australia reported the resistance of *A. duodenale* [188] to pyrantel. However, the data should be interpreted with caution since some confounding variables (small sample size, absence of a placebo group) may have introduced a bias in the results [189]. In addition, it was suggested in a study performed in Pemba Island (Tanzania) that repeated treatments (over a period of 5 years) with MBZ might have selected for drug resistance in hookworms. In this area, the CR and ERR decreased significantly six years after implementation of the control program [82]. However, molecular analysis of the β-tubulin gene at codon positions 200 and 167 of the suspected resistant worms did not identify polymorphisms at these positions [190]. Moreover, the efficacy of MBZ against hookworms was also questioned in another study conducted in Vietnam [69]. Except for the study in Pemba Island, for all other studies assessment of resistance was based solely on FECRT and CR.

Conclusive evidence of drug resistance was not provided by any of these studies due to various factors (study design, diagnostic tests used, etc.) that prevented clear conclusions [67]. In addition, the low sensitivity of the tests used

to assess resistance was an important limitation of these studies. Development of accurate diagnostic tools for monitoring drug resistance is urgently required, which was the impetus for my thesis.

1.7.1. Diagnostic tools for detecting benzimidazole resistance

BZ resistance could be detected by using different tests including the FECRT, *in vitro* tests such as the egg hatch assay (EHA) and molecular assays based on the polymerase chain reaction (PCR).

As previously mentioned, the FECRT which measures changes in faecal parasitic egg count following chemotherapy is a standard biological test widely used in veterinary and human parasitology to assess AH efficacy [73,189]. The FECRT is also used for veterinary parasites as the standard method for measurement and detection of resistance. Nevertheless, with this test, resistance is identified if 25% of the worm population is already resistant [191]. In veterinary parasites, a population is considered to be resistant if the ERR is less than 95% and the lower confidence interval is below 90% [73]. For human STHs, the defined thresholds are different. Indeed, resistance is suspected with an ERR below 70% for *A. lumbricoides* and below 50% for *T. trichiura* and hookworms [74]. This indicator is subject to a considerable number of confounding variables. First of all as mentioned above, resistance could be detected only if resistance is already established in the population. Second of all, the FECRT is mainly influenced by the sensitivity of the diagnostic tests used for the FEC, which may lead to sample bias. (See section on Diagnostic for soil-transmitted helminths).

Thus, some efforts have been made to develop and standardize simple and cost-effective *in vitro* assays as alternative tests [192].

The egg hatch assay (EHA) is an *in vitro* test for monitoring AH resistance. The EHA assesses the ability of thiabendazole (TBZ) to inhibit the hatching of nematode eggs expressed as the dose required to inhibit the hatching of 50% of the eggs (ED_{50} or LD_{50}). In veterinary nematodes the EHA has been standardized and has been defined as when the ED_{50} exceeds 0.1 mg/mL of TBZ the parasitic isolate is considered to be BZ resistant [73]. Since 1999, the development of the EHA for human hookworms has been supported by the WHO [74] and the assays have been applied in several studies that proved its ability to give quantitative and qualitative measurements of the effect of TBZ on egg hatching [193,194]. In the literature it has been described that the EHA could be easily applied in a field situation and it is characterized by the low cost of the procedure in terms of labour and time [195,196]. Unfortunately, the EHA has not been standardized yet for human parasites because of the lack of information on the dose response of know resistant and susceptible field hookworm isolates [197].

Finally, the development of molecular-based tests to detect SNPs associated with resistance has opened new perspectives on anthelmintic resistance research. Molecular tests designed to identify AH resistance in veterinary and human parasites are based on different PCR techniques. In the past, molecular tools for the analysis of AH resistance have been confined to BZ-susceptible and resistant genotypes. Diagnostic PCR allows the binding of the primers to specific

sequence variants [175]. These allele specific tests have been developed for the analysis of the susceptible and resistant genotypes in the β-tubulin gene and could be combined with a PCR restriction fragment length polymorphism (RFLP) procedure [198]. Subsequently, real time PCRs were conducted with diagnostic PCR tests to quantify the allele frequency of the SNP of interest. The assays have been designed for human STHs and tested on *A. duodenale* and *N. americanus,* and on the dog hookworm, *A. caninum* to monitor codon positions 167, 200 and 198 in the β-tubulin isotype 1 gene. In all species, the level of polymorphisms found at all loci was low and, therefore suggested that in the tested samples selection for resistance did not occur [156,199]. Application of real time PCR to the veterinary nematode *H. contortus* and to the filarial parasite *Wuchereria bancrofti* confirmed the sensitivity, reliability and reproducibility of the test on field samples [183,200]. In addition, the pyrosequencing method based on "sequencing by synthesis" has also been developed and applied to human STHs for detecting SNPs in the β-tubulin isotype 1 gene at codon positions 200, 167 and 198 [195]. The pyrosequencing method has several advantages, including accuracy in detecting the frequency of single or multiple SNPs, high throughput, reliability, and the ability to determine proportions of alternative nucleotides at a SNP site in pools of eggs, larvae, or worms [179,183]. Both real time PCR and pyrosequencing are sensitive tools to detect SNPs associated with resistance in the β-tubulin gene. However, the requirement of sophisticated equipment and cost could limit the use of these DNA assays in a field context. In this current thesis we will extensively discuss the application of pyrosequencing DNA assays to

assess SNP frequencies prior to and following ABZ treatment in STH samples collected in the field.

1.8. Current situations in Haiti, Kenya and Panama, endemic countries for STHs

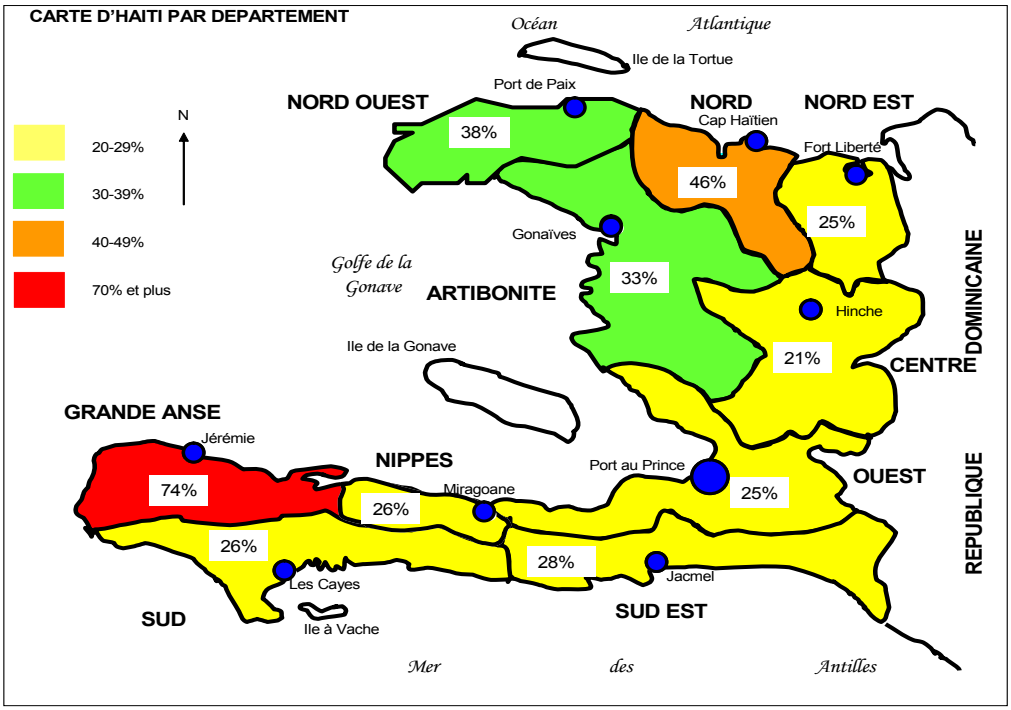
In the literature, studies performed in Haiti, Kenya and Panama have reported on the prevalence of STHs, existing controls programs and monitoring of ABZ resistance.

Haiti is located in the West Indies and occupies the western third of the island of Hispaniola, which is shared with the Dominican Republic. The country is divided into 9 departments that are further divided into 41 "arrondissements" and 133 municipalities (Figure 1.8).

In Haiti, the national program to eliminate LF began in 2001 and was initiated in Leogane. Annual treatment with a combination of DEC and ABZ is distributed through the community level program [201]. Before the implementation of the control program the total prevalence of STHs in school children from this area was estimated at 54.7% [202]. In 2002, a national survey on STHs was conducted in SAC and revealed that 27.3%, 7.3% and 3.8% of children were infected with *A. lumbricoides*, *T. trichiura* and *N. americanus,* respectively [203] (Figure 1.8). Another study conducted in 2004 in four sentinel sites surrounding the locality of Leogane showed a significant decrease in prevalence from 20% to 14.1% for *A. lumbricoides*, from 34 to 14.6% for

T. trichiura and from 11.2% to 2% for hookworms, 9 months after the MDA [204]. However, drug efficacy was not assessed. In the same area, genomic analysis of the β-tubulin gene at codon position 200 of *A. lumbricoides*, *T. trichiura* and hookworms was performed and identified this SNP in *T. trichiura* and hookworms [157,195].

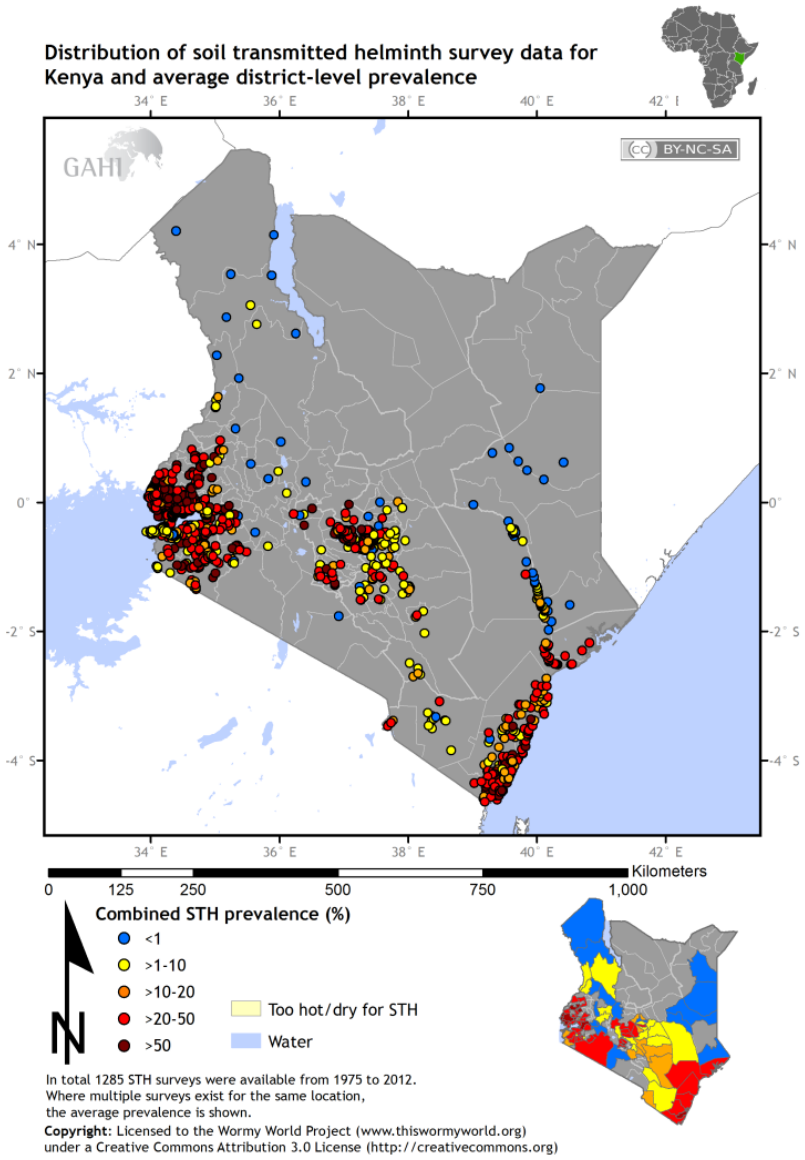
Figure 1. 8. Distribution of soil-transmitted helminths in SAC in Haiti



Source [203]

In Kenya, located in East Africa, a national program for deworming SAC was conducted in 2008 by the Ministries of Education and Health [205]. The prevalence of STH has been mapped (Figure 1.9) and it was estimated that 56.8% of SAC were infected with intestinal parasites. Molecular analysis of adult *A. lumbricoides* and *T. trichiura* β-tubulin gene collected from untreated subjects in Kisumu, revealed the presence of SNP 200 in whipworms [157].

Figure 1. 9. Distribution of soil-transmitted helminths in Kenya



Source: http://www.thiswormyworld.org

Finally, in Panama in the region of Comarca Ngäbe-Buglé, a health and nutrition program was conducted on PSAC, and ABZ was provided as treatment [206]. In samples collected in that region, SNP 200 was identified in *T. trichiura* from treated individuals [157].

There is a growing concern that drug resistance could develop in STHs, and if it is the case it will threaten STH control programs. Low drug efficacies have been extensively reported, especially against whipworms. There is, unquestionably, an urgent need to develop accurate and sensitive tools to detect the distribution and frequency of SNPs associated with resistance.

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CONNECTING STATEMENT I

In the first chapter we reviewed the current knowledge of STH infections and the causative parasites. We also presented the BZ drugs used to control STHs. These drugs reduce the prevalence, intensity and morbidity of infections, however, a reduced or low efficacy of BZ against hookworms and *T. trichiura* has been reported in several studies. In the current situation some tests are available to detect the resistance of STHs to drugs. However, they are either not standardized or not sensitive enough. Thus, there is need to develop accurate tools to detect early genetic changes in the β-tubulin gene. The following chapter presentes our molecular diagnostic tests to detect mutations associated with resistance in the β-tubulin genes of *A. lumbricoides*, *T. trichiura* and hookworms at codon positions 167, 198 and 200. We also optimized the biological EHA on the canine hookworm *A. caninum* and applied it on human hookworms to assess the response to BZs.

CHAPTER TWO

Molecular and biological diagnostic test for monitoring benzimidazole resistance in human soil-transmitted helminths

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Abstract

In endemic countries with soil-transmitted helminths (STHs) mass drug administration with albendazole or mebendazole are being implemented as a control strategy. However, it is well known in veterinary helminths that the use of the same benzimidazole drugs can place selection on the β-tubulin gene, leading to resistance. Given the concern that resistance could arise in human STHs there is an urgent need to develop accurate diagnostic tools for monitoring resistance. In this study we developed molecular assays to detect putative resistance genetic changes in *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms and we optimised an egg hatch assay for the canine hookworm *Ancylostoma caninum* and applied it to *Necator americanus*. Both assays were tested on field samples. The molecular assays demonstrated their reproducibility, and capacity to detect the presence of worms carrying putative resistance-associated genetic changes. However further investigations needed to validate our molecular and biological tests on additional field isolates.

2.1. Introduction

Soil transmitted helminth (STH) infections, caused by the three most important STHs: *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), and *Necator americanus/Ancylostoma duodenale* (hookworms) are a major health problem in developing countries. The number of cases of STHs is estimated to be 1,221 million people worldwide for *A. lumbricoides*, 795 million for *T. trichiura,* and 740 million for hookworms [1]. Among them, 400 million school-age children are infected by STHs, resulting in children being physically and intellectually compromised by anemia, attention-deficit, and learning disabilities. In addition, 44 million pregnant women suffer from severe anemia due to hookworms. These infections are endemic in tropical and sub-tropical regions of the developing world with the greatest burden in sub-Saharan Africa, East Asia, China, India, and South America [2-4]. STH infections are not only responsible for 135,000 deaths annually but also impose a devastating socioeconomic burden on affected communities. It is estimated that 39 million Disability Adjusted Life Years (DALYs) are lost worldwide each year due to STHs [5]. The high prevalence, percentage of cases and intensity of infections rank STHs as one of the major neglected tropical diseases [3,6]. Control strategies through mass drug administration (MDA) programs have been implemented to decrease the prevalence, intensity and morbidity associated with STHs and are based on a periodic distribution of a single dose of the benzimidazole (BZ) derivatives albendazole, or mebendazole [7,8]. Albendazole is the most commonly used benzimidazole (BZ) anthelmintic drug as it shows more activity

against hookworms than other anthelmintics [9]. Regular treatments in endemic countries have shown positive effects on health [10]. However, in veterinary medicine, the widespread and frequent use of BZ drugs has led to extremely high rates of resistance in multiple nematode species in a wide range of hosts [11]. BZ resistance in parasitic nematodes is characterized by single nucleotide polymorphisms (SNPs), which cause amino acid substitutions from phenylalanine (Phe TTC) to tyrosine (Tyr TAC) in β-tubulin at codons 200 or 167 [12,13]. Also, a glutamate to alanine change at codon 198 (Glu198Ala) has occasionally been associated with BZ resistance [14] [15]. In human STHs, there is a growing concern that repeated MDA with the same BZ drugs could exert similar selection pressure on parasites and favor the development of resistance. Some studies have suggested an emergence of drug resistance by reporting a low cure rates (CR) and fecal egg count reductions (FECR) [9,16,17]. However, these major indicators, used for assessing drug efficacy, lack sufficient precision and accuracy to conclude the development of resistance [18-20]. Thus, there is an urgent need to develop better diagnostic tools for detecting BZ resistance in human parasites. We have previously developed DNA based assays to detect the Phe200Tyr SNP associated with BZ resistance in whipworms and roundworms [21]. The objectives of this study were 1) to develop standardized molecular tests for *A. lumbricoides*, *T. trichiura* and *N. americanus* for SNPs in the three critical positions associated with BZ resistance in other nematode parasites, and 2) to optimize the egg hatch assay for assessing the response of hookworms to BZ anthelmintics.

2.2. Material and Methods

2.2.1. Acquisition of parasite material

Molecular assay development and optimization were performed using DNA samples from *A. lumbricoides, T. trichiura* and *N. americanus* collected in areas where mass drug administration (MDA) was not ongoing. DNA from adult *A. lumbricoides* and *T. trichiura* had been extracted previously using the DNeasy blood and tissue extraction kit (Qiagen, Inc, Toronto, ON, Canada) [21].The ability of the molecular assay to detect resistance-associated SNPs in human hookworms was tested by applying DNA from *N. americanus* collected from 28 persons in areas under MDA with albendazole in Haiti and from five children previously treated with mebendazole, on Pemba Island, Tanzania [22] and provided by Dr. M. Albonico to R. Kaplan at the University of Georgia (Table 2.1). Human stool samples were collected as part of a study of MDA for lymphatic filariasis and STH in Haiti, being conducted by Dr. Patrick Lammie of CDC, Atlanta, GA. The samples were collected prior to MDA in Cayes-Jacmel located in the South-east Department of Haiti. Haiti is known to be endemic for STHs, including *N. americanus* [23]. In samples from three adults, the response of hookworm eggs to BZ was assessed by the egg hatch assay (EHA). The optimization of the biological assay was done using both dog and human hookworm parasites. Fresh canine stool samples were collected at a dog pound in Athens, GA. Dogs at the pound carried natural infections of the canine hookworm. In addition, stool samples were provided by TRS Labs, Athens, Georgia from dogs used to maintain a culture of *A. caninum*.

Table 2. 1. Origin and treatment history of N. americanus DNA samples used in the molecular assays for human hookworms. Adult and larvae samples from Pemba Island were recovered from five infected children [22]. In Haiti, eggs were recovered from 28 persons.

	Pemba Island	Haiti
Specimen	DNA from adult worms and larvae	DNA from eggs
Sample size	2 DNA samples of individual adult worms 7 DNA samples, each of 50 larvae	27 DNA samples each of 10 eggs
Treatment history	Post-Treatment Mebendazole	Post-Treatment Albendazole
Round of treatment	13	Unknown

2.2.2. Ethical approval

Ethical approval (study 2535) was obtained (Dr. Patrick Lammie) from the Centers for Diseases Control and Prevention, Atlanta, Georgia, US and included the collection of stool samples, examination of stool samples for helminth eggs and DNA analysis of helminth eggs. Oral informed consent was obtained from all human adult participants and from parents or legal guardians of minors.

2.2.3. Wild type and mutant type plasmid constructs

From a previous study, genetic markers, validated with control plasmids, were constructed for position 200 in *A. lumbricoides* and *T. trichiura* [21]. In this work, we also investigated codons 167 and 198 of the β-tubulin gene, involved in resistance in other parasitic nematodes [15]. Diagnostic tests were optimized by using control plasmids to investigate codons 167 and 198 in β-tubulin of *A. lumbricoides*, *T. trichiura*, *N. americanus*, and codon 200 in *N. americanus*. For each parasite species, wild type plasmids (WT), and mutant type plasmids

(MT) carrying a mutation either at codon 167, 198 or 200, generated by site directed mutagenesis, were produced as described previously [21]. WT and MT plasmids contained the β-tubulin sequences of interest (with or without mutations). Primers for wild type (Table 2) and mutant type plasmid (Table 3) were designed in the exonic regions of genomic DNA, for *A. lumbricoides* (FJ501301.1, GenBank acc. number), and *T. trichiura* (AF034219.1 GenBank acc. number) and in exonic and intronic regions for *N. americanus* (EF392851, GenBank acc. number). All PCR reactions contained: 2 µl 10×PCR buffer, 1 µl [50 mM] $MgSO_4$, 1 U Platinum Taq DNA Polymerase High Fidelity, 1 µl of sense and antisense primers [10 µM], 1 µl dNTP mix [10 µM], 2 µl template DNA and distilled H_2O to 20 µl. Negative controls, without DNA, were included. The polymerase chain reaction (PCR) conditions remained the same for all parasites, except for annealing temperatures that varied according to the species and the position amplified (Table 2.2 and 2.3). All PCR reactions included an initial denaturation at 94°C for 45 s followed by 35 cycles of 94°C for 45 s, a specific annealing temperature (see Table 2), for 45s, an elongation at 68°C for 1 min and a final extension at 68°C for 5 min. Wild type and mutant type amplified fragments were ligated into a TOPO-TA cloning vector (Invitrogen™, Life Technologies, Burlington, ON) and subsequently sequenced at the McGill University/Genome Quebec Innovation Centre, Montreal, Quebec.

Table 2. 2. Species and SNP specific primers for wild type plasmids of the three STH β-tubulins. T°ann, primer annealing temperature

Codon	Species	Primer sequences (5'-3')	T°ann (°C)
167	*A. lumbricoides*	Forward: ACTCGCTTGGTGGAG Reverse: CAACCAACTGATGAACGGAC	58.4
	T. trichiura	Forward: CCTCGGCGGCGGAACTG Reverse: GGTGGACTGACAGAGTTGC	56.4
	N. americanus	Forward: TGACTGTCTCCAGGTAATTCG Reverse: CGATGATACGCGGGATG	58
198	*A. lumbricoides*	Forward: CCGTGAAGAATACCCCGACA Reverse: TATGTGGGATTTGTAAGCTTCAG	58.7
	T. trichiura	Forward: TTTCAGATACAGTTGTAGAACC Reverse: CAAATGATTTAAGTCTCCG	57
	N. americanus	Forward: GTTTCCGACACTGTGGTTGAG Reverse: AGTTCGTTACTAGCCAGCTCACC	59
200	*N. americanus*	(same as position 198)	

Table 2. 3. Species specific primers for mutant type constructs in the three STH β-tubulins. SNP-Fwd, forward primer mutated for a single nucleotide; SNP-Rev, reverse primer mutated for a single nucleotide; T° ann, primer annealing temperature.

Codon	Species	Primer sequences (5'-3')	T°ann (°C)
167	*A. lumbricoides*	Forward: GCACTCGCTTGGTGGAG Reverse: CAACCAACTGATGAACGGAC SNP-Fwd: GCTCGTACTCAGTTGTTCCATC SNP-Rev: GATGGAACAACTGAGTACGAGC	58.4
	T. trichiura	Forward: CCTCGGCGGCGGAACTG Reverse: GGTGGACTGACAGAGTTGC SNP-Fwd: CAACTTATAGTGTCGTTCCGTC SNP-Rev: GACGGAACGACACTATAAGTTG	58.5
	N. americanus	Forward: TGACTGTCTCCAGGTAATTCG Reverse: CGATGATACGCGGGATG SNP-Fwd: CGTTATCCGTTGTACCCTC SNP-Rev: CAACGGAATACGAGGACAT	59
198	*A. lumbricoides*	Forward: TGAAGAATACCCCGACA Reverse: CTGAAGCTTACAAATCCCACATA SNP-Fwd: ACACCGATGCAACCTTC SNP-Rev: GAAGGTTGCATCGGTGT	58.7
	T. trichiura	Forward: GTTTCAGATACAGTTGTAGAACC Reverse: CAAATGATTTAAGTCTCCG SNP-Fwd: ACACGGACGCAACATTC SNP-Rev: GAATGTTGCGTCCGTGT	50
	N. americanus	Forward: GTTTCCGACACTGTGGTTGAG Reverse: GATTCAGATCTCCATATGTTGGG SNP-Fwd: AGATGCGACCTTCTGTATTGATAATG SNP-Rev: CATTATCAATACAGAAGGTCGCATCT	57
200	*N. americanus*	Forward: GTTTCCGACACTGTGGTTGAG Reverse: GATTCAGATCTCCATATGTTGGG SNP- Fwd:AGATGAGACCTACTGTATTGATAA TG SNP-Rev: CATTATCAATACAGTAGGTCTCATCT	58

2.2.4. Diagnosis of resistance-associated mutations in *A. lumbricoides*, *T. trichiura and N. americanus*

Mutated and susceptible alleles were detected for all parasites by pyrosequencing. To optimize our assays, the sense, antisense and sequencing primers required for pyrosequencing were designed by PyroMark Assay Design Software (Qiagen, version 2.0, Inc, Toronto, ON, Canada) (Table 4). The PCR reactions contained 5 µl 10×PCR buffer, 2 µl [50 mM] $MgSO_4$, 1 U Platinum Taq DNA Polymerase High Fidelity, 1 µl of sense and antisense primers [10 µM], 1 µl dNTP mix [10 µM], 3 µl template DNA and distilled H_2O to 50 µl. Antisense primers were biotinylated at their 5'end to generate single-stranded template using streptavidin beads (Amersham, Biosciences, Piscataway, NJ). The amplification conditions were: 94°C for 45 s followed by 50 cycles of 94°C for 45 s, 59°C for 45 s and 68°C for 1 min and a final extension at 68°C for 5 min. Sequencing primers (Table 2.4) were used for SNP analysis in the pyrosequencer.

Table 2. 4. Allele-specific primers and sequencing primers used for pyrosequencing reactions for the three STH β-tubulins. Seq primer, sequencing primer; T° ann, primer annealing temperature.

Codon	Species	Primer sequences (5'-3')	T°ann (°C)
167	*A. lumbricoides*	Forward: CCGTGAAGAATACCCCGAC Reverse: GGTGGACTGACAGAGTTGC Seq primer: ACCCCGACAGAATCATGAGCTCG	59
	T. trichiura	Forward: CCTCGGCGGCGGAACTG Reverse: GGTGGACTGACAGAGTTGC Seq primer: TGACCGAATTATGACAACT	59
	N. americanus	Forward: CCGGATCAGGAATGGGAAC Reverse: GGCTCAACCACAGTGTCG Seq primer: GATAGAATCATGTCCTCGT	59
198	*A. lumbricoides*	Forward: AGGTTTCTGATGTGGTGTTGGA Reverse: CAAATGATTTAAGTCTCCG Seq primer: GGTTGAGAACACCGAT	59
	T. trichiura	Forward: AGGTTTCAGATACAGTTGTAG Reverse: CAAATGATTTAAGTCTCCG Seq primer: GGTAGAGAACACGGACG	59
	N. americanus	Forward: GTTTCCGACACTGTGGTTGAG Reverse: GATTCAGATCTCCATATGTTGGG Seq primer: GTTGAGAATACAGATG	58
200	*N. americanus*	Forward: GTTTCCGACACTGTGGTTGAG Reverse: GATTCAGATCTCCATATGTTGGG Seq primer: GTTGAGAATACAGATG	58

2.2.5. Molecular assay applied to field hookworm samples

Hookworm DNA from pools of eggs or larvae, and individual worms were amplified using the same primers, and PCR conditions described above. All PCR products were checked on agarose gel before performing genotyping of the Phe200Tyr SNP with the pyrosequencer.

2.2.6. Allele frequency and sequence analysis

Plasmids and hookworm genotypes were obtained by pyrosequencing and the allele frequencies of each SNP were determined by the AQ module in the PSQ^{TM}96 Single Nucleotide Position Software (Biotage^{TM}AB, Charlottesville, VA). Conventional Sanger sequencing was also performed for each plasmid and chromatograms resulting from sequencing were analysed with Sequencher (version 5.0, Ann Arbor MI). WT and MT plasmids sequences were aligned using CLUSTAL W on Geneious (Version 5.5.6: www.geneious.com).

2.2.7. Egg Hatch Assay for hookworms

Before being applied to human hookworms, the egg hatch assay was optimized using dog hookworms (*A. caninum*). One gram of fecal material was used to perform an egg count and the rest was stored under anaerobic conditions to avoid larval development until egg isolation was performed. To isolate the eggs, one gram of feces was suspended in distilled water and the mixture was poured into a tube, through surgical gauze to remove large debris. After centrifugation for 10 min at 300 g the supernatant was discarded and a saturated sucrose solution (Sp. Gr. 1.27) was added. The sediment was thoroughly mixed with the sugar solution until fully resuspended, and then additional saturated sucrose solution was added to form a slight positive meniscus. Before centrifugation, a cover slip was placed on the top of the tube and then after centrifugation the tube was left for 10 minutes before removing the cover slip. The cover slip was rinsed with distilled water to remove the eggs, and the number of eggs recovered was counted using low power microscopy (40X magnification).

Residual sucrose was removed by successive washing with distilled water, and centrifugation. Additional one-gram samples were used to isolate more eggs as needed. After all eggs for a given sample were recovered, the isolated eggs were vortexed and 3 separate 20 µl aliquots were taken to count the numbers of eggs present in the sample. The sample volume was then adjusted to yield a concentration of approximately 1 egg per microlitre.

An agar-based assay system was used as previously described [24]. A stock solution of thiabendazole (Sigma- Aldrich, St Louis, MO) was serially diluted in DMSO (Sigma- Aldrich, St Louis, MO) by two-fold dilutions. Ten drug solutions ranging from 0.8 µg/ml to 0.0015125 µg/ml were produced. In the assay plates, the first two wells of each row served as non-drug controls (2 µl DMSO), the third well contained the lowest concentration chosen, and the last well the highest. Then 200 µl of 2 % agar (Bacto Agar ,VWR Becton Dickinson Sparks, MD) was added to each well containing the drug or DMSO. Assay plates were allowed to cool so that agar would harden and then sealed with parafilm and placed in sealed zip-lock bags in the refrigerator at 4 °C until used. At the time of the assay, plates were removed from the refrigerator and allowed to warm to room temperature. Forty microlitres, containing approximately 40 hookworm eggs were then added to each well and the plates placed into an incubator at 29°C. Plates were incubated for 16 hours, 18 hours or 45 hours, and assays were defined as completed when 95 to 100% of eggs had hatched in the control wells. At the end of the assay each well was scored by counting the number of larvae (hatched eggs) and unhatched eggs. All assays were performed in triplicate for each drug

concentration tested. Various experimental parameters were tested (summarized in Table 2.5) to assess their impact on the dose response, and to determine the optimal conditions for assaying human hookworms under lab field conditions. The qualitative parameters tested included the duration of the assay, the composition of the plate (agar-based vs liquid-based), the drug concentration, the storage condition of the stool containing eggs, the *A. caninum* isolates (either lab isolates of *A. caninum* [TRS strain] or field isolates, the addition of antifungal amphotericin B [10 μg/10 ml] (Fisher, Pittsburgh PA), and finally the addition of Lugol's iodine solution, after the incubation, to stain eggs and larvae, and sodium thiosulfate to destain the background.

2.2.7.1. Experiment A: *A. caninum* isolates, duration of the assay and plate composition.

In this experiment two assays were set up and in each of them five drug concentrations (from 0.8 to 0.003125 μg/ml) were tested. For both assays, the plates used were made using either an agar or a liquid matrix. Experiment A was split into two assays, in the first (A-1), 40 μl of egg suspension from lab isolates was added to each well filled either with agar or liquid. Eggs and larvae were scored in each well after 18 hours of incubation, and a second time after 45 hours of incubation. In the second assay (A-2), the conditions remained the same except that field isolates of *A. caninum* were used.

2.2.7.2. Experiment B: Storage conditions of stool-containing egg

Fresh stool samples from dogs infected with the lab isolate of *A. caninum* were stored under different anaerobic conditions: The first consisting of putting a fresh fecal sample in a specialized zip-lock plastic bag where air was removed through a port using a vacuum pump (Reynolds Handi-Vac®, Walmart, Athens, GA) and left at room temperature until needed (Experiment B-1). In the second, fresh stool samples were placed in two containers of 50 ml with 15 glass beads and water, and shaken to disrupt the stool. Each container was then filled completely with water and shaken again to produce anaerobic conditions. One container was placed at room temperature (Experiment B-2) and the other at 4°C (Experiment B-3). Prior to the anaerobic storage of the stool sample, an egg count was performed to determine the number of eggs per gram. Three days after the anaerobic storage, three assays were set-up (one for each storage condition). Six drug concentrations were tested (from 0.8 to 0.003125 μg/ml). All plates used were agar-based and the plates were incubated for 18 hours.

2.2.7.3. Experiment C: Staining and destaining of eggs and larvae

In this experiment, eggs recovered from the non-refrigerated stool sample (as in Experiment B-2) were used. Again, six drug concentrations were tested (from 0.8 to 0.003125 μg/ml) and the incubation time was 18 hours. To evaluate if staining the eggs and larvae made them easier to count, 10 μl of 50 % Lugol's iodine solution followed by 20 % sodium thiosulfate were added to each well at the end of the assay.

2.2.7.4. Experiment D: Addition of an antifungal solution, Amphotericin B

In this experiment, prior to adding the egg suspension to the assay wells, 90 µl of the amphotericin B [10 µg/10 ml] antifungal solution was added to each 1ml egg suspension. All conditions of the assay remained the same as in Experiment C.

2.2.7.5. Experiment E: Optimized assays

In this experiment agar plates were prepared under the same conditions that would be used in the field to assay human hookworms. In all plates seven drug concentrations were tested (from 0.8 to 0.003125 µg/ml). Eggs were recovered from fresh fecal material obtained from dogs infected with the *A. caninum* lab isolate (TRS strain). Two assays were performed using the same conditions as are described in Experiment D. In both assays egg hatching was scored at 16 hours and 18 hours.

Table 2. 5. Conditions applied to each egg hatch assay. [1]Experiment A was split into two assays (A-1 and A-2), in experiment A-1 eggs were recovered from feces of dog infected with lab isolates of *A. caninum* (TRS strain) while in experiment A-2, eggs were recovered from feces of dogs infected with field isolates of *A. caninum.* [2]Eggs recovered from stool were stored under anaerobic conditions prior the assays.[3]Eggs were recovered from fresh stool samples prior the assays.[4]The temperature given refers to the storage temperature of the feces and not the incubation temperature of the assay. [5]Staining and distaining solutions applied into wells at the end of the assay to facilitate the scoring. [6]Antifungal solution added to the egg solution prior to the assay. In assays from all experiments drug concentrations tested were from 0.8 μg/ml to 0.003125μg/ml. RT, room temperature; N/A, not applicable.

	End point (hrs)	**Solutions**	**Storage type and temperature**
Experiment A[1]	18 45	N/A N/A	N/A N/A
Experiment B-1[2]	18	N/A	Anaerobic in Ziploc bag at RT[4]
Experiment B-2[2]	18	N/A	Anaerobic in air tight container at RT[4]
Experiment B-3[2]	18	N/A	Anaerobic in air tight container at 4°C
Experiment C[2]	18	50% Lugol's iodine[5] + 20% sodium thiosulfate[5]	Anaerobic in air tight container at RT
Experiment D[2]	18	Amphotericin B[6]+ 50% Lugol's iodine + 20% sodium thiosulfate	Anaerobic in air tight container at RT
Experiment E[3]	16	Amphotericin B + 50% Lugol's iodine + 20% sodium thiosulfate	N/A

2.2.8. Application of the egg hatch assay to human hookworms

The assays were performed using the protocol optimized with *A. caninum.* Assay plates containing agar and drug were prepared in the laboratory at McGill University and transported to the field site in Haiti. Plates were prepared with seven drug concentrations (from 0.8 to 0.003125 μg/ml) (as in Experiment E).

Hookworm eggs were recovered from fresh fecal samples as described above. Aliquots of egg suspension containing amphotericin B were disposed into each well, with all assays performed in duplicate. The average number of eggs allocated to each well of assays 3010019, 3010022 and 3010028 was 39, 11, and 10, respectively. Plates were incubated for 16 hours at room temperature at approximately 29°C and staining and destaining solutions were added to each well at the termination of the assays.

2.2.9. Statistical analyses

Data from egg hatch assays were analysed by Logit models that describe the statistical model [25] and its application by fitting the data of each individual egg hatch assay and showing the relationship between the expected proportions of unaffected eggs (not hatching) at each concentration and the log concentration [26]. The dose response data were obtained by a non linear regression curve. The drug sensitivity was calculated as the LC_{50}, which is respectively the drug concentration required to decrease the number of eggs hatching to 50% of that observed in the control wells (no drug). The LC_{50} values and 95% confidence intervals were calculated in GraphPad Prism® (version 5.0 for Windows, GraphPad software, San Diego, CA).

2.3. Results

2.3.1. Development of molecular markers for each species

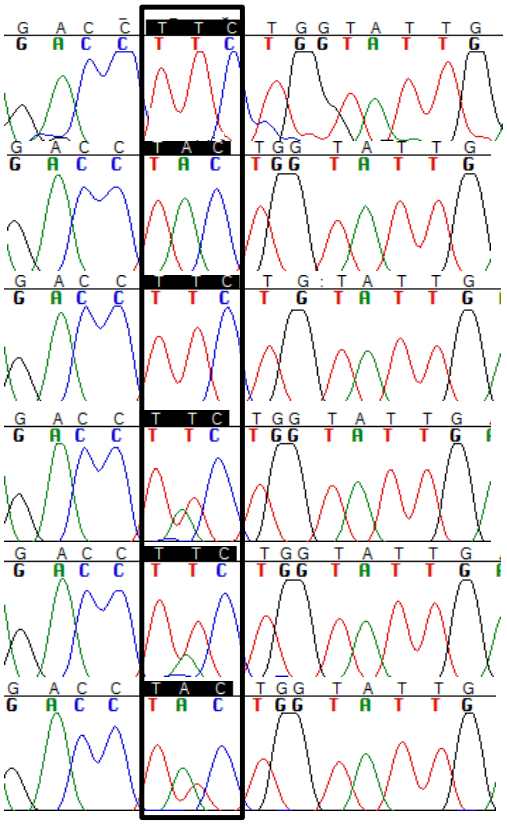
Sequencing of the WT and MT plasmids clearly showed that we had generated the desired mutations at codons 167 and 198 for the three species and at codon 200 for *N. americanus*. From the pyrosequencing we obtained distinct genotype profiles illustrated by pyrograms for WT and MT (Figure 2.1). For codons 167 and 198, WT plasmids identified the homozygote susceptible alleles "T" and "A", respectively. The MT plasmids identified, at codon 167 and 198, the homozygote resistant alleles "A" and "C", respectively (data not shown). Those results confirmed that all diagnostic tests were efficient as they could identify easily and clearly the susceptible and resistant-type alleles for *A. lumbricoides*, *T. trichiura* and *N. americanus*. From a previous study [21] the Phe200Tyr SNP diagnostic assays using pyrosequencing were applied to *A. lumbricoides* and *T. trichiura* field samples. The results obtained validated the reproducibility and efficacy of those assays on field samples. WT and MT plasmids served as positive controls when running pyroseqencing assays for positions 167, 200 or 198. In this study, the pyrosequencing assay was used to screen *N. americanus* samples from Pemba Island and Haiti (Table 2.1). All samples were tested for the Phe200Tyr SNP. In samples from Pemba Island, the mean allele frequency of the susceptible allele "T" was 100% in individual adult worms (n=2). In pool samples (n=7 pools; each pool of 50 larvae) the mean percentage of the susceptible allele "T" was 90% and the mean allele frequency of the resistance allele "A" was 10%. The same samples had previously been screened for the Phe200Tyr SNP by allele-specific

PCR and real-time PCR. In hookworm samples from a treated area in Haiti (n=27 pools of 10 eggs), two samples failed the PCR amplification. Among the remaining 25 samples, the mean allele frequency of the susceptible allele was 64% and the mean of the resistance-type allele was 36% (Table 2.6). Genotypes for some of these samples were confirmed by conventional Sanger sequencing. In samples carrying the resistance-type allele (TAC), (chromatograms D, E, F), a double peak at the second nucleotide (T and A) of the codon 200 was always detected and for each of these samples, and the pyrosequencer never showed an allele frequency of the resistance-type allele (TAC) higher than 65%. Chromatograms of the control plasmids were included to compare with those of the field samples (Figure 2.1).

Table 2. 6. Frequency of the susceptible allele "T" and the resistance allele "A" detected by the pyroseqencer in 25 pools, each of 10 *N. americanus* eggs, collected in Haiti. *Percentages below 7% may be misleading due to pyrosequencing errors on pooled samples.

	Allele Frequency (%)	
Pool IDs	T	A
1	40.0	60.0
2	61.9	38.1
3	61.1	38.9
4	95.6	4.4*
5	77.6	22.4
6	63.2	36.8
7	61.9	38.1
8	42.9	57.1
9	54.6	45.4
10	93.1	6.9*
11	53.5	46.5
12	62.9	37.1
13	90.8	9.2
14	70.7	23.3
15	43.7	56.3
16	73.6	26.4
17	89	11
18	34.8	65.2
19	68.1	31.9
20	60.6	39.4
21	77.5	22.5
22	72.3	27.7
23	58.4	41.6
24	46.8	53.2
25	22.7	77.3

Figure 2. 1. Chromatograms resulting from conventional Sanger sequencing of *N. americanus* control plasmids and field samples of the β-tubulin gene around codon 200. A**:** The chromatogram of the wild type (WT) plasmid showed the presence of a single peak (T) at the second nucleotide of the codon (TTC), this characterizes the homozygous susceptible type (TTC/TTC). B: The chromatogram of the mutant type (MT) plasmid revealed the presence of the codon associated with resistance to benzimidazoles (TAC). Chromatograms C to F show PCR products of pooled egg samples from Haiti, C (pool ID# 10 in Table 6) shows a single peak "T" at the second position of the nucleotide whereas the sequences shown in chromatograms D (pool (ID# 11 in Table 6), E (ID# 12 in Table 6), F (ID# 1 in Table 6) have two peaks "T" and "A" at the same position.



2.3.2. Egg hatch assay

In addition to molecular assays, a biological assay was developed for *A. caninum* and then adapted to human hookworms in order to detect resistance to BZ drugs. In all assays, eggs and larvae were counted in each well and the relationship between drug concentration and egg hatching were established using a logistic regression model for dose-response, which permitted the calculation of LC_{50} (Figure 2.2). Data are summarized in Table 2.7.

2.3.2.1. Experiment A

In this experiment, three parameters were evaluated: the hookworm isolates, the duration of the assay and the use of an agar-based assay vs. a liquid-based assay. No differences in dose response were seen between the two *A. caninum* isolates; at the same drug concentrations the proportions of eggs hatching were similar. However, agar-based assays were easier to score than liquid-based assays, and the data were more consistent. For both assays, 45h of incubation made the counting of eggs and larvae difficult due to fungal growth and the dryness of the agar plate. The long incubation did not increase the number of larvae that hatched; the scoring of eggs and larvae at 18h remained the same as at 45h (data not shown).

2.3.2.2. Experiment B—D

In these three experiments, we tested the impact of the following parameters on the dose response: the storage conditions of the stool containing eggs (anaerobic conditions and temperature), the addition of Lugol's iodine,

destainer, and amphotericin B. Experiment B-1, showed that the storage of the fecal material in specialized Zip lock type bags, left at room temperature, was not an appropriate anaerobic environment as it did not prevent the hatching of eggs. Thus, it was not possible to perform the EHA using these samples. We observed a difference in egg hatch response to thiabendazole between assays performed with refrigerated and non-refrigerated eggs stored in an tight air container. Indeed, the LC_{50} of the assay obtained from Experiment B-3 with refrigerated eggs (0.0258) was significantly different from the LC_{50} of the assays from Experiment B-2 and D where non-refrigerated eggs were used (0.0522 and 0.0496 respectively) as the 95% confident intervals of the LC_{50} did not overlap. In contrast, the assays performed with non-refrigerated eggs (Experiments B2 and D), the 95% confidence intervals for LC_{50} overlapped, demonstrating no significant difference in dose response. In Experiments C and D, addition of the antifungal solution, Lugol's iodine and sodium thiosulfate showed qualitative improvement as they facilitated the scoring of unhatched eggs and larvae (Figure 2.2A and Table 2.7).

2.3.2.3. Experiment E

All conditions shown to quantitatively and qualitatively improve the EHA were applied in this experiment. By 16h, larvae had hatched in the control wells and were motile. The same observation was found in the previous experiments after 18h incubation. Sixteen hours of incubation was therefore considered as the end point of the assay. Using these assay conditions there was no significant difference between the LC_{50} (overlapping 95% confidence intervals (CIs)) in the

two assays. Overall the replicates of the assays showed consistent results (Figure 2.2B).

Table 2. 7. LC_{50} for thiabendazole in the egg hatch assay with *A. caninum*. The 50% lethal concentration was determined after 16h or 18h of exposure of *A. caninum* eggs to thiabendazole (see text). In experiment E, two assays were conducted under the same conditions.

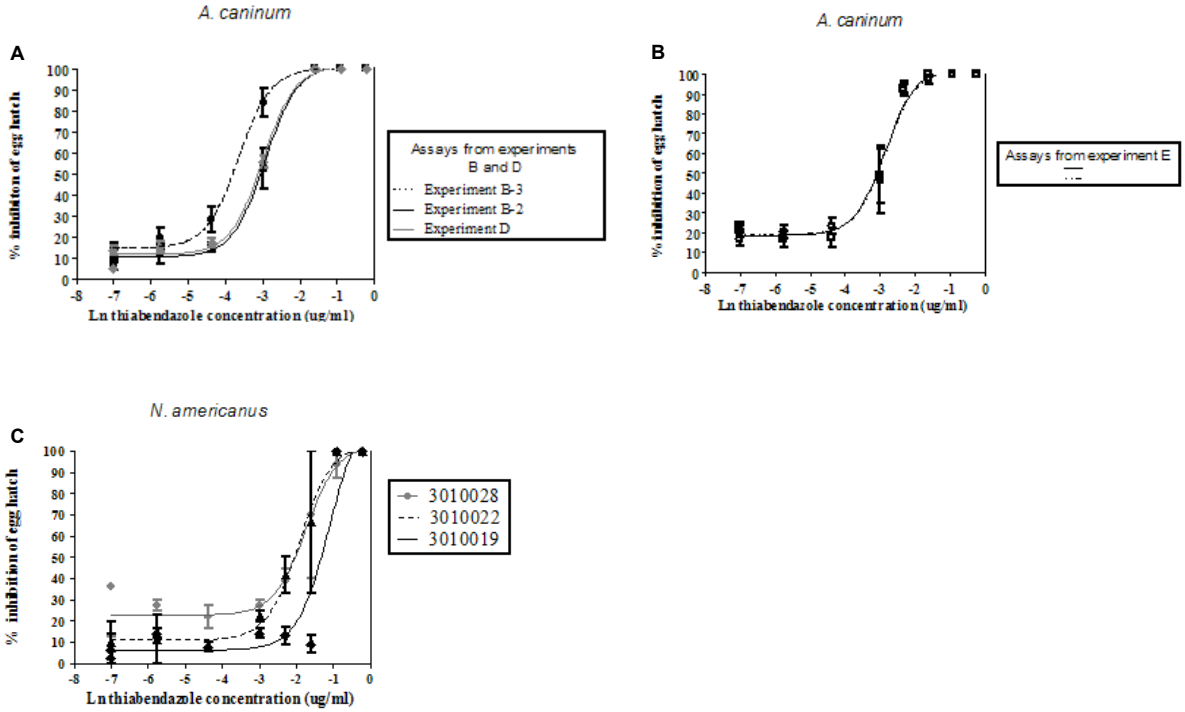
Assay from experiments:#	LC_{50} (µg/ml) (95% Confidence interval)
B-3	0.0258 (0.0205-0.0324)
B-2	0.0522 (0.0415-0.0655)
D	0.0495 (0.0394-0.0622)
E	0.0547 (0.0435-0.0687)
	0.0357 (0.0284-0.0448)

2.3.3. Egg hatch assay (EHA) on human hookworms

Application of the EHA on field *N. americanus* eggs recovered from stool samples of three study participants produced egg hatch responses that were less sensitive to thiabendazole. The LC_{50} of the assays with *N. americanus* ranged from 0.1237 to 0.2566 µg/ml, compared with results obtained with *A. caninum* (LC_{50} ranged from 0.0258 to 0.0547 µg/ml). There was no inhibition of hatching at lower concentrations, and this shift in sensitivity meant that the concentration range tested was not high enough to achieve a maximal effect (Figure 2.2C). However, it was still possible to calculate the LC_{50}, and these were not significantly different (the 95% CIs for the LC_{50} overlapped) among the three *N. americanus* samples. The major issue encountered when attempting to perform

the assays were the low egg counts in the *N. americanus* positive participants, which limited the number of eggs we could recover and use. Less than optimal egg numbers caused us to use fewer eggs per well and to perform the assays in duplicate, rather than triplicate. This led to high variability in the data, which reduced the precision of the assays.

Figure 2. 2. Dose-responses curves for *A. caninum* and *N. americanus* to thiabendazole. Dose responses of A. caninum in egg hatch assays with thiabendazole, after 18h (A) obtained from three experiments B-2, B-3 and D where different conditions were used. In experiment B-3 eggs in anaerobic condition were refrigerated prior to the assay while in experiment B-2 they were kept at room temperature. In experiment D, antifungal solution was added to the egg suspension prior to the assay. Panel B shows the dose responses of A. caninum to thiabendazole after 16h, obtained from experiment E. Panel C, shows the dose responses of field N. americanus, after 16h exposure, obtained from three subjects naturally infected in Haiti (3010028, 3010022 and 3010019) (C). All curves were obtained with the logit model; the dots represented the observed data obtained in all assays.



2.4. Discussion

The development of accurate tools to detect, at an early stage, the possible development of resistance in human parasites is now a priority with large scale MDA programmes being launched for STHs. During the past ten years, there has been some suggestion of benzimidazole selection occurring in filarial nematodes [27] human hookworms [17,22] and in human whipworms [21]. However, in these studies evidence of genetic selection was not directly linked with resistance. Nevertheless, diagnostic tools based on SNP markers for codon 200 have been developed and validated through pyrosequencing and the real-time PCR [28]. In this study, two molecular markers, for codon 167 and 198, were developed in *A. lumbricoides*, *T. trichiura* and *N. americanus* and, in addition, for codon 200 in *N. americanus*, using the pyrosequencing method. Wild type and mutant type control plasmids were genotyped and these showed two distinct genotypic profiles for each parasite species and for each SNP position. Thus, for the three most important human STHs, we have developed molecular makers for the three positions in the β-tubulin gene, which based on experience in veterinary nematodes, are likely to be critical for benzimidazole resistance.

The pyrosequencing method, based on "sequencing by synthesis", has several advantages, including accuracy in detecting the frequency of single or multiple SNPs, high throughput, reliability, and the ability to determine proportions of alternative nucleotides at a SNP site in pools of eggs, larvae, or worms [27,29,30]. The goal in developing such assays was to have reproducible procedures for SNP detection that could be used to monitor for possible resistance

selection in MDA programmes for human STHs. In our protocol, primer sequences for each reaction were determined and tested against plasmid constructs, each step in the procedure has been tested on field samples of human STH and the procedures are detailed. We are aware that one of the major limitations to the use of this molecular technique, in a field context, could be the cost of the pyrosequencer. However, once the samples are collected, the genotyping could be done in labs where the equipment is available. Our molecular techniques are therefore suitable for resistance monitoring on field samples, where multiple samples are to be collected. The accurate assessment of SNP frequencies can then be performed in a short period of time.

We also applied the assay of the Phe200Tyr SNP on *N. americanus* from areas treated with albendazole and mebendazole for many years. In samples from Pemba Island, DNA from two individual adult worms showed the susceptible SNP and in a pool of hookworm larvae the percentage of the susceptible SNP was close to 100% even though those samples had been recovered from children in which mebendazole sensitivity had been lower than those found in previous years [16]. However, our genotyping result was consistent with the previous molecular analyses performed by conventional Sanger sequencing and real time PCR methods [28] [28]. Due to a lack of sufficient DNA, analyses of codons 167 and 198 were not performed. However they have been investigated by others [28] who failed to detect the presence of the resistance-associated SNP at either position. As suggested by these authors, further analyses on the β-tubulin gene are necessary to determine if it is possible to link the low BZ efficacy with other molecular

changes in β-tubulin. In pools of 10 eggs/person of *N. americanus* recovered from 28 persons in areas in Haiti under MDA, the resistance-type SNP (codon 200) was identified (frequency = 36%). According to the genotype frequency obtained in each pool, we could estimate (assuming Hardy-Weinberg equilibrium) that the egg population was mainly composed of homozygous susceptible and heterozygous resistant eggs, though some homozygous resistant eggs may be present. Because BZ resistance has been shown to be a recessive trait in several nematode parasites of animals [31], it is likely that overall the population will appear susceptible, but that any increase in the frequency of the resistance allele, with further treatments, could lead to the appearance of a resistance phenotype in the population. Genotypes were confirmed by conventional Sanger sequencing.

The pyrosequencing method has an error rate in determining SNP frequency of approximately 5 - 7% [28].This means at low allele frequency (≤ 7%) the estimate may be unreliable. Taking this error rate at low SNP frequencies into account, the resistance allele was identified in 92% of the samples and confirmed by conventional Sanger sequencing. The results indicate that the proportion of the resistance-type allele in the total population was moderately high. The finding of resistance-associated polymorphisms in *N. americanus* from a treated area raises the question whether further repeated treatment may cause an increase in the frequency of the resistance allele. To answer this question it will be important to perform longitudinal studies where egg samples will be collected before treatment, and after several rounds of treatment, and to verify if, in the same host population, the allele frequency increases after treatment. To understand better the

correlation between both parameters (treatment and the resistant allele frequency), it would be interesting to mathematically model any change in resistance allele frequency after a given number of rounds of treatment, assuming an initial resistance allele frequency as determined in this or other studies. This kind of model has been applied to filarial nematodes and been used to predict the spread of the resistance by considering different parameters [32].

In this study, we also described the development of an in vitro egg hatch assay for dog hookworms, which can be used to assess sensitivity to a benzimidazole drug. We investigated the dose response of *A. caninum* to thiabendazole, the best analogue of the benzimidazole class for measuring resistance in vitro. Before applying the same assay to human hookworms, we optimized it with canine hookworms, which are close to human hookworms in terms of phylogeny, and dose response to anthelmintics [33,34]. In veterinary parasites, the EHA has been standardized, even if from one study to another some variations remain [35]. For instance it has been clearly defined for some veterinary nematodes that when the LC_{50} is in excess of 0.1 μg/ml of thiabendazole that the parasite isolate is considered to be benzimidazole resistant [36]. Since 1999, the development of the EHA in human hookworms has been supported by the WHO and the assays have been applied in several studies that proved its ability to give quantitative and qualitative measurements of the effect of thiabendazole on hatching of the eggs [24,37,38]. However, the assay has not been standardized because of the lack of resistant isolates of human hookworms [38]. The attempt to optimize our assay was based on quantitative and qualitative

criteria, and on the suitability of the assay for human hookworm eggs collected in the field. The EHA described in this study was adapted from the protocol described by Kotze and colleagues [24].

First of all, it was observed that increasing the duration of incubation from 16 h to 18 h did not have any effect on the LC_{50}, but longer incubation (45 h) had an adverse effect on the dose response as the scoring of egg hatching was made difficult. In addition, the use of an agar-based assay was better than a liquid-based assay for two reasons: it is more practical to prepare and transport plates with the solid-phase of agar [38], as our final objective was to use the EHA in the field, and agar-based plates could be more easily standardized [38]. In addition, the agar-based assay showed less variation in the drug concentrations affecting egg hatching, compared with the liquid based assay [24]. However, in a recent paper on dose-response assays, it was shown that both, agar and liquid-based assays have the same sensitivity to discriminate known resistant and sensitive *Haemonchus contortus* isolates [38]. In this study, we also compare the impact of fecal storage conditions and temperature on the egg hatch response. Based on our data we recommend anaerobic storage of eggs at room temperature since it is unknown how storage at 4°C may affect the fitness of the eggs and their ability to develop. Furthermore, refrigeration may not be available in the field. Additional work is needed to assess the value of refrigerating the eggs to delay development prior to commencement of an EHA. We also observed that more stringent washing of the egg preparation reduced bacterial growth, and that the use of Lugol's iodine improved qualitatively scoring egg hatching, but had no significant

impact on the dose response. Thus, in our study we determined important qualitative and quantitative parameters that have an impact on the egg hatch response assessment in *A. caninum* and showed the reproducibility of the test once optimized.

The suitability of the conditions of the EHA, in a field context was another factor considered. Indeed, the temperature of incubation (29°C) of the assays was set according to the ambient temperature that could be reached in a field laboratory in a tropical country where electricity shut downs may be frequent or incubators might not be available. Another important consideration was the low cost of the procedure in terms of labour and time. Agar-based plates could be made beforehand and stored at 4°C or at room temperature for 78 days without showing any change in dose response [38]. Finally the EHA was applied in the field on *N. americanus* using the same conditions that had been established for *A. caninum*. Unfortunately, a small sample size and low intensity of *N. americanus* infection prevented conclusions on the in vitro susceptibility of *N. americanus* in the area investigated. Beyond this limitation, two additional ones prevented the standardization of the egg hatch assay on human hookworms. Indeed, as previously highlighted [38], the lack of information on the dose response of know resistant and susceptible field isolates make decisions on the susceptibility of hookworm samples difficult. In this study, even though the three assays with *N. americanus* showed a lower sensitivity to thiabendazole compared with *A. caninum* and *N. americanus* used in other studies [24,37,38], this does not mean that the field isolates used in this study were resistant, as there is no data

available, so far, on the response of a fully characterized field resistant strain as a reference. In addition, the lack of data on the LC_{50} for human hookworms was also a challenge for attempts to standardize the assay. The range of drug concentrations used was not sufficient to achieve a maximal effect. As suggested by Kotze and colleagues, a broader range of drug concentration should have been tried in order to achieve maximal drug response [38]. Finally, to validate this assay as a diagnostic tool to detect resistance, it will be interesting to correlate the LC_{50}, the frequency of the resistance alleles and response to treatment in terms of egg count reduction.

2.5. Acknowledgments

We are grateful to Dr Patrick Lammie from the Centers for Diseases Control and Prevention for his contribution to the study. We thank Dr. Thomas Streit from Notre Dame University for the logistic support in Haiti. We also thank the staff members of the Hôpital Sainte-Croix in Léôgane for their commitment and their support: Dr. Luccene Desir, Wesly Pierre, Dardith Desire, Wilman Metelus, Rose-Guerda Louis-Charles, Wilfrid Jean, Jean Makatu Innocent, Shiler Emile, Eudson Romulus, Max Rosemond and Michelet Leriche. We also thank Dr. Marco Albonico for providing hookworm DNA samples, and Dr. John McCall and TRS Labs Inc. for providing canine stool samples containing *A. caninum* eggs.

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CONNECTING STATEMENT II

In chapter II, we developed molecular diagnostic tests for *A. lumbricoides*, *T. trichiura* and hookworms that were validated with control plasmids. We assessed the frequency of the β-tubulin gene polymorphism at position 200 in hookworm samples from Haiti, collected in an area under treatment with ABZ. The results revealed the presence of the resistance allele at a moderate frequency suggesting that repeated treatments with the same drug might increase the frequency of this polymorphism. To determine whether ABZ influences the frequency of polymorphisms identified in the β-tubulin gene of *A. lumbricoides*, *T. trichiura* and hookworms we assessed the frequency of the SNPs at positions 167, 198 and 200 before and after ABZ treatment in samples collected in Haiti, Kenya and Panama. We also compared the frequency of the polymorphisms with albendazole respon each parasite. The results are presented in Chapter 3.

CHAPTER THREE

Association between response to albendazole treatment and β-tubulin genotype frequencies in soil-transmitted helminths

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Abstract

Background: Albendazole (ABZ), a benzimidazole (BZ) anthelmintic (AH), is commonly used for treatment of soil-transmitted helminths (STHs). Its regular use increases the possibility that BZ resistance may develop, which, in veterinary nematodes is caused by single nucleotide polymorphisms (SNPs) in the β-tubulin gene at positions 200, 167 or 198. The relative importance of these SNPs varies among the different parasitic nematodes of animals studied to date, and it is currently unknown whether any of these are influencing BZ efficacy against STHs in humans. We assessed ABZ efficacy and SNP frequencies before and after treatment of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm infections.

Methods: Studies were performed in Haiti, Kenya, and Panama. Stool samples were examined prior to ABZ treatment and two weeks (Haiti), one week (Kenya) and three weeks (Panama) after treatment to determine egg reduction rate (ERR). Eggs were genotyped and frequencies of each SNP assessed.

Findings: In *T. trichiura*, polymorphism was detected at codon 200. Following treatment, there was a significant increase, from 3.1% to 55.3%, of homozygous resistance-type in Haiti, and from 51.3% to 67.8% in Kenya (ERRs were 49.7% and 10.1%, respectively). In *A. lumbricoides*, a SNP at position 167 was identified at high frequency, both before and after treatment, but ABZ efficacy remained high. In hookworms from Kenya we identified the resistance-associated SNP at position 200 at low frequency before and after treatment while ERR values indicated good drug efficacy.

Conclusion: Albendazole was effective for *A. lumbricoides* and hookworms. However, ABZ exerts a selection pressure on the β-tubulin gene at position 200 in *T. trichiura*, possibly explaining only moderate ABZ efficacy against this parasite. In *A. lumbricoides*, the codon 167 polymorphism seemed not to affect drug efficacy whilst the polymorphism at codon 200 in hookworms was at such low frequency that conclusions cannot be drawn.

Author Summary

The soil-transmitted helminths (STH) *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Ancylostoma duodenale* and *Necator americanus* are endemic in many tropical countries. Regular treatment with albendazole or mebendazole is the major means for controlling STHs. However, repeated treatment with the same class of benzimidazole anthelmintics has caused resistance in veterinary parasites, characterized by mutations at either codon 200, 167 or 198 in the β-tubulin gene. There is a concern that resistance may develop in human STH. Drug efficacy and mutation frequencies were assessed in *T. trichiura*, *A. lumbricoides* and hookworms collected in Haiti, Kenya and Panama prior to and after albendazole treatment. In *T. trichiura* from Haiti and Kenya, a significant increase of the frequency of the mutation at codon 200 was identified after treatment and drug efficacy was mediocre. Against *A. lumbricoides*, albendazole efficacy was good, even though the frequency of a mutation at codon 167 was relatively high, suggesting that, in this nematode, the codon 167 polymorphism does not impact efficacy. In hookworms, the mutation at codon 200 was identified, but at low frequencies and the response to albendazole was good. We conclude that monitoring for possible resistance in control programmes should be undertaken.

3.1. Introduction

Ascaris lumbricoides (roundworm), *Trichuris trichiura* (whipworm) and *Necator americanus / Ancylostoma duodenal*e (hookworms) are the most common species of soil-transmitted helminths infecting humans worldwide. More than a billion people are infected with at least one species and 300 million are estimated to have severe infections with more than one of these parasites [1]. These infections are endemic in tropical and sub-tropical regions of the developing world and are associated with poverty, lack of clean water, and poor sanitation [2]. School-age children are the most at risk of infection with STHs and early childhood infections contribute significantly to debilitation [3]. Infected children can be malnourished and experience stunting growth and intellectual retardation, with cognitive and educational deficits [1]. Because of all these characteristics and according to estimates by the World Health Organization (WHO) [4], STHs are included in the group of the so-called neglected tropical diseases (NTDs). The main intervention to control STH infections at a community level is based on periodic mass drug administration (MDA) of the benzimidazole (BZ) anthelmintic (AH) drugs, albendazole (ABZ) or mebendazole (MBZ) [5], that reduce the prevalence and intensity of infections [6]. However large-scale chemotherapy programmes with these drugs have the potential to exert selection pressures on the causing parasites, which may favour the development of drug resistance. Recently, expansion of MDA programmes for STHs have highlighted the need to monitor for the possibility that resistance may develop [7,8]. This development would have important adverse consequences on the benefits provided by

deworming programmes [9,10]. BZ resistance in other parasitic nematodes is caused by a single nucleotide polymorphism (SNP) in the β-tubulin gene at codon positions 200 (T → A), 167 (T → A), or 198 (A → C) [11,12,13]. The frequency and relative importance of these different SNPs varies among the nematode species studied to date [13,14,15]. Molecular markers have been developed to identify SNPs in *A. lumbricoides*, *T. trichiura* and hookworms and wild-type and mutant-type control plasmids have been constructed to obtain the genotype profiles of "susceptible-type" parasites (which do not have mutations at position 200, 167 and 198) and "mutant-type" parasites (that contain mutations in one of these three positions) [16]. The codon 200 polymorphism has been identified in *T. trichiura* populations collected from untreated subjects in Kenya and from treated subjects in Panama [17], and in hookworms collected in Haiti in an area periodically treated with ABZ [16]. In the present report, our aim was to investigate the field efficacy of ABZ against STH infections in countries where polymorphism in β-tubulin had previously been identified, and to assess the frequency of each SNP prior to, and after ABZ treatment within each country and for each of the three main nematode species.

3.2. Materials and Methods

3.2.1. Study Design and Study Areas in Haiti, Kenya and Panama

Three cross-sectional studies were carried out between 2008 and 2010 in three countries located in different geographical areas, including Haiti in the

Caribbean, Kenya in East Africa and Panama in Central America. These studies were integrated into the following control and evaluation programmes of ABZ use: a national health programme to fight lymphatic filariasis (LF) and intestinal worms in Haiti (in collaboration with the Hôpital Ste. Croix and the Centers for Disease Control (CDC)); school health and nutrition programmes in Kenya, and health and nutrition programmes in Panama. Thus, each study included a treatment with ABZ, stool examinations, and genotyping of the β-tubulin gene in eggs collected before and after ABZ treatment to assess the drug efficacy against STHs and to examine at each time point the frequency of possible SNPs associated with ABZ resistance in nematodes of veterinary importance. The study designs in Kenya, Panama and Haiti (Figure 3.1) were different because each study was part of a separate national and local MDA programme. Thus, different protocols were applied rather than a purposely designed multi-study site single protocol.

3.2.2. Study in Haiti

The study in Haiti included stool collections prior to, and after a drug treatment given in 2009. In Haiti, the study sites were located in the West and Southeast Haitian departments known to be naive for MDA with ABZ. Individuals from five endemic communities who reached the inclusion criterion (older than two years old) were randomly selected. All potential participants were informed by a trained community leader of the purpose and methods of the study and gave their oral consent (in the case of children, consent was obtained from a parent). A total of 353 stool samples were collected and analyzed prior to ABZ

treatment. Treatment was then distributed to all people of each community (400 mg ABZ and 6 mg/kg diethylcarbamazine (DEC)). ABZ was supplied as a donation from GlaxoSmithKline (GSK). Participants in the study were not observed during the treatment administration. However, the compliance to treatment was evaluated post-treatment by a questionnaire and relied on self-reported information of each participant, and on pre and post-treatment egg counts. Samples from participants who were not treated with ABZ were not included in the analysis. Follow-up faecal samples (n=317) were collected two weeks after the drug treatment.

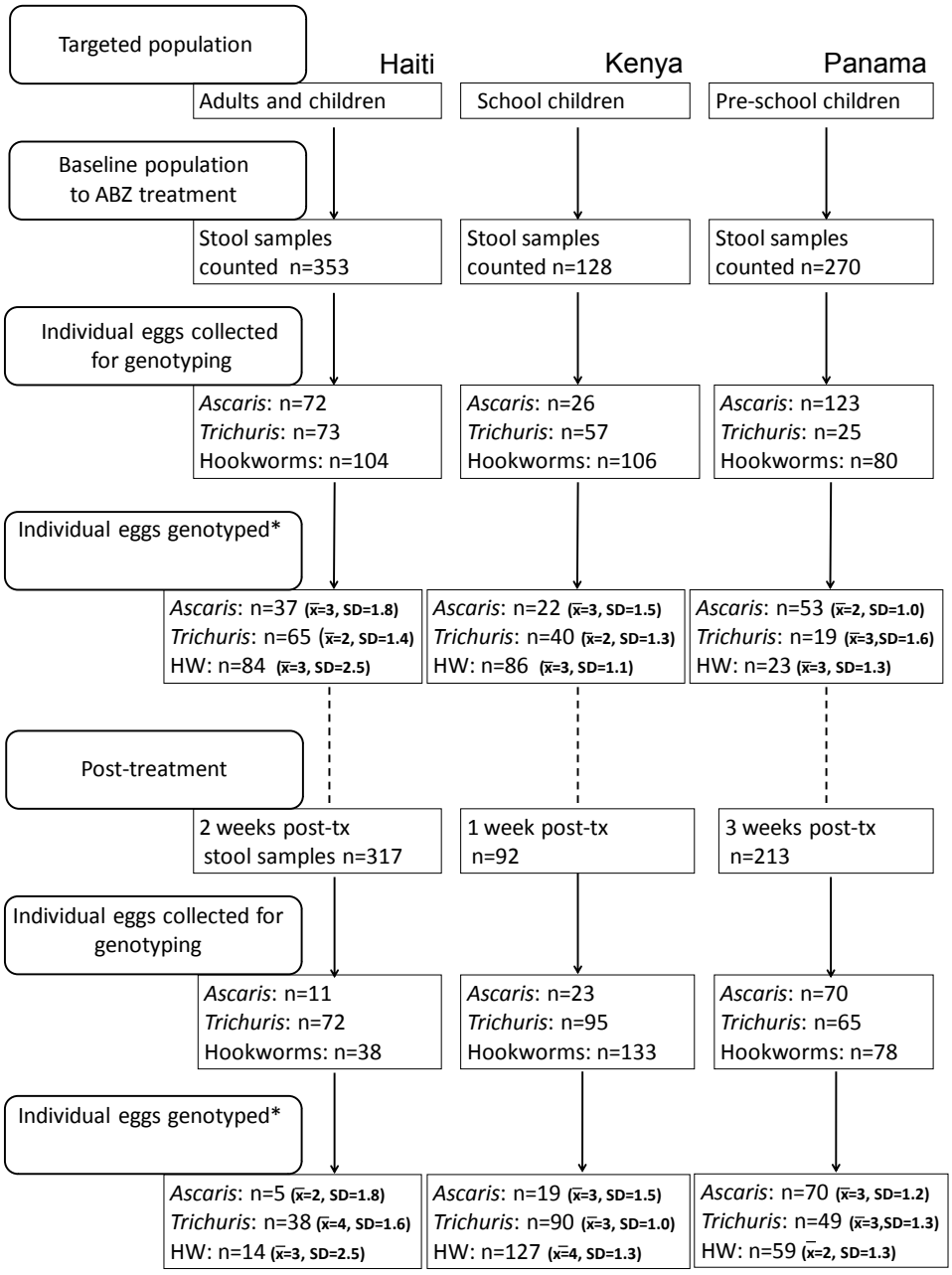
3.2.3. Study in Kenya

School-aged children from two schools located in Kwale District were enrolled in the study. Although the schools were selected because at the time of the study they were not involved in any other STH deworming programmes, pupils had received ABZ and DEC six month prior to our study for LF treatment. Stool samples were collected prior to ABZ treatment in both schools (n=128) and then all children received 400 mg ABZ under the supervision of the school teachers. The drug was provided by the Kenyan Ministry of Health and was manufactured by GSK. Seven days later, a follow-up collection was undertaken (n=92). In one of the schools, the stool collections before and after ABZ treatment were done over two consecutive days and the results averaged.

3.2.4. Study in Panama

The field study was conducted in the region of Comarca Ngäbe-Buglé [18]. Stool samples were collected from pre-school children at different time points over a period of 16 months from July 2008 to October 2009. Two treatments with ABZ, as a suspension (200 mg: one to two years of age; 400 mg: three to five years of age) were distributed, once in 2008 and the second. nine months later in 2009 [19]. The drug was provided by the Panamanian Ministry of Health. In the present study, we only analyzed the faecal samples collected prior to treatment and three weeks after treatment in 2009 since the baseline prevalence for STHs was very low in the 2008 samples. Prior to this second ABZ treatment, stool samples were collected from 270 pre-school aged children and follow-up samples were available from 222 children. Children were observed while received the ABZ treatment, and only samples from treated children were included in the analysis.

Figure 3. 1. Flow chart of the study designs in Haiti, Kenya and Panama. *The number of eggs genotyped is different from the number of eggs collected. This is due to failures in DNA extraction, PCR amplification or Pyrosequencing. The dashed lines mean that the connection between the two boxes is not direct. $\bar{x}$, representes the mean of eggs sampled per host and SD=standard deviation.



3.2.5. Ethics Statement

In the three studies, all instances of consent were informed. Ethical approval for Haiti was obtained from the Institutional Review Board of the Centers for Diseases Control and Prevention, Atlanta, Georgia, US (Dr. Patrick Lammie), and the Ethics Committee of the Hôpital Ste Croix, Haiti and included the collection of stool samples, examination of stool samples for helminth eggs and DNA analysis of helminth eggs.

Informed consent was obtained from all adult participants and from parents or legal guardians of all minors. A parent or legal guardian gave consent in every case of child participation. Based on past experience, it was likely that some people in the communities would not be able to read. A waiver of written informed consent on the basis that the research presented no more than minimal risk of harm to the subjects and involved no procedures for which written consent is normally required outside the research context in this setting, was requested and approved. The use of oral consent was previously approved by the IRB. Subjects were offered a written copy of the IRB approved consent form. The contents of the approved consent form were explained to each household, following which verbal consent was obtained. The reader of the consent form and a witness signed a copy of the form to indicate the subject's agreement.

The study in Kenya was approved by the Kenya Medical Research Institute (KEMRI) Ethical Review Committee. The informed consent process, which was approved by the KEMRI IRB specifically stated that the study was included in the

national programme on surveillance of disease control and followed established government procedure. The school heads organized meetings with their parent teacher association to obtain agreement for the project. An information sheet was provided. It was emphasized that the participation in the study by children was voluntary and that they may refuse to participate. All children were treated with ABZ and praziquantel in the study. Finally, all samples were anonymised. As part of a national control programme, informed consent was not documented for each individual. The content of the study was verbally explained in detail to parents/guardians, teachers and children from each school. It was done orally as it was necessary to have this explained in local languages. The use of oral consent was previously approved by the IRB. A parent or legal guardian gave oral informed consent in every case of child participation.

The study in Panama was approved by the McGill University Review Board in Canada, the Instituto Conmemorativo de Gorgas and local indigenous leaders in Panama. Written informed consent was obtained from primary caregivers for their own participation as well as that of their children. They were provided with an explanation of the study, its significance, and of participant requirements and rights. They were given an opportunity to ask questions in Spanish and in the local language. A parent or legal guardian gave consent in every case of child participation.

3.2.6. Stool Sample Procedure and Examination

In all studies and for each intervention (before and after ABZ treatment), labelled containers were distributed to each participant and collected the following morning from the community leader in Haiti, schools in Kenya, and each participant's home in Panama. Three diagnostic techniques: McMaster, Kato-Katz, and FLOTAC were used to identify STH eggs and determine the number of egg per gram (epg) in the faecal material collected before and after ABZ treatment.

In Haiti we used a modified McMaster technique on each collected sample. One gram of faeces was suspended with water and the solution was stirred until it was completely broken apart. The mixture was poured through surgical gauze into a centrifuge tube. After centrifugation for 10 min at 15,000 rpm, the supernatant was poured off and the tube containing the sediment was filled with saturated sucrose solution and then gently stirred. After 10 min, an aliquot of the flotation fluid from the upper surface of the solution was transferred into each compartment of a McMaster chamber. The eggs were counted in both chambers using a low power objective (×10). The number of epg of faeces was obtained by multiplying the total number of eggs counted in the two chambers by 50 [20].

In Kenya, the McMaster (as described above) and Kato-Katz techniques [21] were used on samples from one school and Kato-Katz alone was used in the other school. For Kato-Katz, the number of eggs counted was multiplied by 24 to obtain the epg [21].

In Panama, the identification of eggs and the assessment of epg were performed using Kato-Katz [21] and FLOTAC [22,23], as previously described [19].

One of the primary objectives of the study was to assess the frequency of SNPs associated with resistance to ABZ (seen in the veterinary nematode *Haemonchus contortus*) in the β-tubulin gene of STHs collected from untreated and treated subjects. Thus, from the three studies, eggs from positive subjects were recovered using a saturated sucrose solution with centrifugation, and recovered eggs were preserved in 70% alcohol until use for molecular analysis.

3.2.7. Examination of the β-tubulin Gene and Assessment of SNPs

3.2.7.1. DNA Isolation from Individual Eggs of STHs

Eggs previously preserved in 70% alcohol were washed in distilled water and separated by species under a dissecting microscope. Individual eggs were isolated with a 10 µl pipette and then placed into a PCR tube. Genomic DNA of each individual egg was then extracted according to a protocol elaborated by Lake and colleagues [24] and adapted to STHs. One hundred millilitres of lysis buffer (50mM KCl, 10mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% Nodidet P-40, 0.45% Tween 20, 0.01% gelatine) was previously prepared, and an aliquot of 1 ml of this lysis buffer was taken to which 10µl proteinase K (10 mg/ml) and 10 µl β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA) were added. The solution of lysis buffer with proteinase K and β-mercaptoethanol was placed at -20°C for one week. Individual eggs were placed into a labelled PCR tube and 15 µl of the

mixture was added. The tube was subsequently frozen at -80°C for 30 min and then incubated at 60°C for 2h. This procedure of freezing and heating the egg solution helps the digestion of the eggshell to facilitate DNA extraction. The proteinase K was inactivated by heating to 94°C for 15 min.

3.2.7.2. Genotyping of β-tubulin Gene Positions 167, 198 and 200 in *A. lumbricoides*, *T. trichiura* and Hookworms

After DNA extraction, all samples were subjected to PCR to amplify small fragments surrounding the SNP at codon positions 167, 198, and 200 in the β-tubulin genes of *A. lumbricoides* (FJ501301.1, GenBank acc. number), *T. trichiura* (AF034219.1 GenBank acc. number) and hookworms (EF392851.1 GenBank acc. number). All primers used for genotyping with the Pyrosequencer were designed with the PyroMark Assay Design Software (Qiagen, version 2.0) to amplify single egg DNA. To allow for nested PCRs on single eggs, some primers were different from those used in our previous study [17] in which the DNA concentrations were higher. For PCR, 2 µl of lysate was used as template in a 20 µl reaction containing 2 µl 10×PCR buffer, 1 µl [50 mM] $MgSO_4$, 1 unit Platinum Taq DNA Polymerase High Fidelity, 1 µl of sense and antisense primers [10 µM] (details in Supplementary Table S1, Appendix B), 1 µl dNTP mix [10 µM], and distilled water to 20 µl. PCR reactions were performed with the following cycling parameters: 94°C for 3 min followed by 30 cycles of 94°C, 59°C for 45s and 68°C for 1 min with a final extension step at 68°C for 5 min. A second PCR reaction using the earlier PCR product as template was necessary to visualize the product

on an agarose gel. The reaction contained 5 µl 10×PCR buffer, 2 µl [50 mM] $MgSO_4$, 1 unit Platinum Taq DNA Polymerase High Fidelity, 1 µl of sense and antisense primers [10 µM], 1 µl dNTP mix [10 µM], and distilled water to give a final volume of 50 µl. The same primers were used for each species amplified except for the antisense primer for codon position 198-200 of *A. lumbricoides* (5' CAGATGTCGTACAAAGCCTCATT 3', position). For genotyping, all antisense primers were biotinylated at their 5'end as described [17]. The amplification conditions were 94°C for 3 min followed by 40 cycles of 94°C, 59°C for 45s and 68°C for 1 min with a final extension step at 68°C for 5 min. Sequencing primers for SNP analysis were used to genotype all PCR products at the codon positions 167, 198 and 200 in a Pyrosequencer (Biotage AB, Charlottesville, VA, USA) (Supplementary Table S1, Appendix B). Parasites genotyped did not necessary come from the same subjects sampled before and after treatment. Eggs were extracted from as many hosts as possible, though finding sufficient number post-treatment was restricted by low egg density. Therefore all eggs, from the same community or school, were pooled, before analysis of genotypes of individual eggs. The number of eggs isolated and genotyped are presented in the flow-chart (Figure 3.1).

3.2.8. Statistical Analysis

The treatment efficacy on *A. lumbricoides*, *T. trichiura* and hookworms was evaluated by the egg reduction rate (ERR) for each diagnostic method applied in each country: McMaster (in Haiti, and Kenya), Kato-Katz (In Kenya and Panama) and FLOTAC (in Panama). The ERR was calculated, at the group level, as the

ratio of the difference between the arithmetic mean of the pre- and post-treatment faecal egg count (FEC) to the pre-treatment arithmetic mean, expressed as a percentage, i.e. ignoring individual variability [25]. The negative individuals at baseline were still sampled at the post-treatment collection and the resulting data were included for the calculation of ERR. Uninfected subjects were included in the mean of the FEC. Confidence intervals of each ERR estimate were determined using a bootstrap resampling method (with replacement) over 10,000 replicates in R (version 2.15.0, Vienna Austria, http://www.R-project.org).

Genotype frequencies of SNPs at positions 167, 198 and 200 in *A. lumbricoides*, *T. trichiura* and hookworms obtained at the pre-treatment collections were compared with the genotype frequencies of the same SNPs obtained at the post-treatment collections using Fisher's exact test within GraphPad Prism (GraphPad software, San Diego, CA, USA).

Deviation from Hardy-Weinberg equilibrium (HWE) was analyzed for the β-tubulin gene at position 200 in *T. trichiura* using Arlequin version 3.1 software [26], where the p-value was calculated based on the Markov-chain method [27]. Deviations from the HWE were not determined for *A. lumbricoides* or hookworm for reasons explained below. When a departure from HWE was observed in *T. trichiura*, we estimated the maximum likelihood frequency of a null allele at position 200. This estimate was calculated using an Expectation-Maximization Algorithm of Dempster and colleagues [28] (EM Algorithm, http://132.206.161.123/em.html).

3.3. Results

3.3.1. Genotype Frequencies for the β-tubulin Gene in *T. trichiura*

In *T. trichiura,* the three codon positions 167, 198, and 200 were found to be polymorphic in samples collected from untreated and treated subjects in Haiti, Kenya, and Panama.

In Haiti we analyzed 65 individual *T. trichiura* eggs from 30 untreated subjects and 38 from 14 treated subjects. We recorded 11% and 47% experimental failure (includes DNA extraction, PCR amplification and Pyrosequencing) in pre- and post-treatment samples, respectively. Before treatment the T → A SNP at codon position 200 (SNP200) was identified at low frequency; 3.1% of individual eggs genotyped were homozygous resistance-type (AA) and 23.1% were heterozygous (TA). After treatment, there was a significant increase in the frequency of the homozygous resistance-type, from 3.1% to 55.3% ($p<0.001$), and a statistically significant decrease of the homozygous susceptible-type, from 75.4% to 21.1% ($p<0.0001$) (Figure 3.2A). The A → C SNP at codon position 198 was also found and showed a statistically significant change in frequency following treatment. However, the changes at codon 198 were less pronounced than at codon 200. After treatment, there was a significant increase of homozygous resistant-type from 3.1% to 13.2% ($p<0.001$), and a significant decrease of homozygous susceptible-type from 73.8% to 63.2% (Figure 3.3).

In Kenya, 40 individual *T. trichiura* eggs from 20 untreated subjects and 90 eggs from 31 treated subjects were genotyped. We recorded 30% and 5%

experimental failure in pre- and post-treatment samples, respectively. Only the codon 200 SNP was polymorphic. The same trend observed in Haiti was seen with a statistically significant increase, from 51.3% to 68.5% (p=0.019) of homozygous resistance-type, and a significant decrease, from 48.6% to 21.4% (p=0.019) of homozygous susceptible-type after treatment (Figure 3.2B).

Figure 3. 2. Genotype frequencies of the β-tubulin gene position 200 in *T. trichiura* from Haiti and Kenya. Genotype frequencies of *T. trichura* collected in Haiti (A) and in Kenya (B); Number of individual *T. trichiura* eggs genotyped according to the available material, in Haiti was 65 in the untreated group (pre-Tx) and 38 in the treated group (post-Tx), in Kenya was 40 in the untreated group and 90 in the treated group. Sequences were diploid, TT indicates the homozygous susceptible-type TTC/TTC, TA the heterozygous TTC/TAC and AA, the homozygous resistance-type TAC/TAC; Tx=treatment, *Indicates a significant difference ($p<0.001$) in genotype frequency between the pre- and post treatment groups. P-values were obtained by Fisher's exact test.

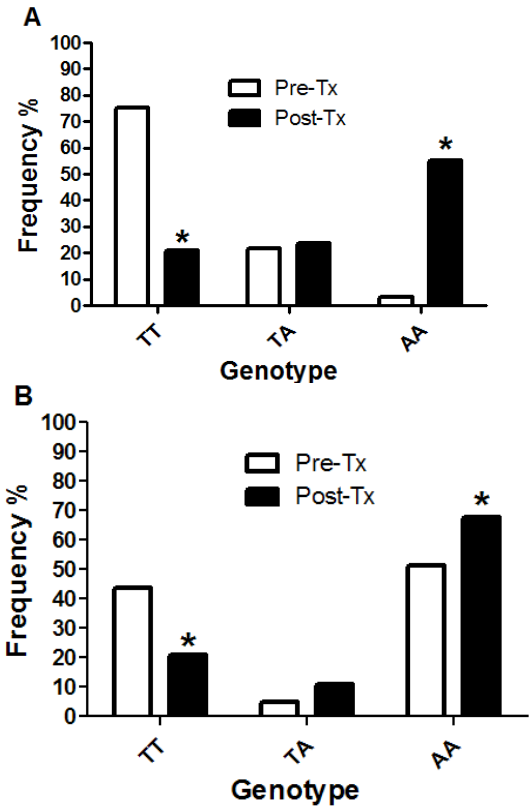
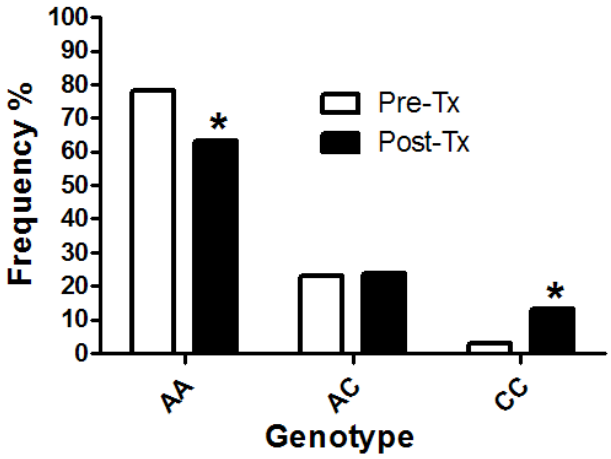


Figure 3. 3. Genotype frequencies of the β-tubulin gene position 198 in *T. trichiura* from Hati before and after ABZ treatment. Genotype frequencies of *T. trichura* collected in Haiti. The number of individual *T. trichiura* eggs genotyped, according to the available material, was 65 in the untreated group (pre-Tx) and 38 in the treated group (post-Tx). Sequences were diploid, AA indicates the homozygous susceptible-type GAA/GAA, AC the heterozygous GAA/GCA and CC, the homozygous resistance-type GCA/GCA; Tx=treatment, *Indicates a significant difference ($p<0.001$) in genotype frequency between the pre- and post treatment groups. P-values were obtained by Fisher's exact test.



In Panama we genotyped 19 "pre-treatment" *T. trichiura* eggs from 10 subjects, which were collected 9 months after an earlier treatment, and 49 post-treatment eggs from 21 subjects collected 3 weeks after this second ABZ treatment. We observed homozygous resistance-type (78.9%) and homozygous susceptible-type (21.1%), at codon 167, in the pre-treatment collection. For codons 198 and 200, 84% of the pre-treatment *Trichuris* egg samples failed the PCR amplification and therefore it was not possible to assess the genotype frequencies for these positions. At the post-treatment collection, the codon 167 polymorphism was still present in the treatment survivors as homozygote resistance-type in 16.3% of individual eggs genotyped. For codons 198 and 200,

we observed a predominance of homozygous susceptible-type genotypes, 97.6% and 88.1% respectively; and a low frequency of heterozygous, 2.4% (for each position) and homozygous resistance-type, 9.5% (for codon 200).

3.3.2. Genotype Frequencies of β-tubulin Gene Positions 167, 198 and 200 in *A. lumbricoides*

In *A. lumbricoides*, the codon position 167 of the β-tubulin gene was polymorphic in parasites collected from untreated and treated subjects in Haiti, Kenya and Panama, whereas the codon positions 198 and 200 were monomorphic. The SNP at position 167 identified was confirmed by conventional Sanger sequencing and real time PCR (data not shown).

In Haiti, we genotyped 37 individual *A. lumbricoides* eggs from 13 untreated subjects and five eggs from three treated subjects. We recorded 49% and 28% experimental failure in pre- and post-treatment samples. Prior to treatment, homozygous resistance-type (40%) and heterozygous (60%), at codon 167, were present in the population; however, only heterozygotes were detected after treatment.

In Kenya, 22 individual *A. lumbricoides* eggs from 6 untreated subjects and 19 eggs from 4 treated subjects were genotyped. We recorded 15% and 17% experimental failure in pre- and post-treatment samples, respectively. The predominant genotype frequency identified was the homozygous resistance-type (72.7%) at codon 167. After treatment, there was no significant difference in the homozygous resistance-type frequency; however, as observed in *A. lumbricoides*

collected in Haiti, at codon 167 there was a statistically significant increase of heterozygotes from 4.5% to 21.1% ($p<0.001$), and also a significant decrease of homozygous susceptible-type from 22.7% to 5.3% ($p <0.001$).

In Panama, 53 individual eggs were genotyped from 28 untreated subjects and 70 eggs from 20 treated subjects. We recorded 57% of experimental failure only in pre-treatment samples. As seen previously in Haiti and Kenya, the most abundant genotype at codon 167 was homozygous resistance-type (97.7%). In this population the lowest genotype frequency was identified as homozygous susceptible-type (2.3%). After treatment, the percentage of both genotypes did not change significantly (96.7% and 3.2%, respectively) (Table 3.1).

Table 3. 1. Genotype frequencies of β-tubulin position 167 in *A. lumbricoides* before and after ABZ treatment. [1]TT= homozygous susceptible-type TTC/TTC, [2]TA= heterozygous TTC/TAC, [3]AA= homozygous resistance-type type TAC/TAC. [4]Tx= treatment. The numbers in parentheses indicate the number of individual eggs genotyped. The letters (a,b) indicate significant difference ($p<0.001$) between the genotypes of the pre- and post treatment groups.

	Frequency (%)					
	Haiti		Kenya		Panama	
Genotypes	**Pre-Tx[4] (n=37)**	**Post-Tx (n=5)**	**Pre-Tx (n=22)**	**Post-Tx (n=19)**	**Pre-Tx (n=53)**	**Post-Tx (n=70)**
TT[1]			22.7[a]	5.3[a]		
TA[2]	60	100.0	4.5[b]	21.1[b]	2.3	3.2
AA[3]	40		72.7	73.7	97.7	96.8

3.3.3. Genotype Frequencies of β-tubulin Gene Positions 167, 198 and 200 in hookworms

In hookworms, the codons 167 and 198 of the β-tubulin gene were monomorphic in all samples genotyped from Haiti, Kenya and Panama. Codon

200 polymorphism (TAC) was detected in 2 eggs collected in Kenya. In Kenya, 86 individual eggs from 28 untreated subjects and 127 eggs from 34 treated subjects were genotyped. We recorded 19% and 4% experimental failure in pre- and post-treatment samples, respectively. In the pre-treatment collection we identified homozygous resistance-type at low frequency (2.3%) and a predominance of homozygous susceptible-type (97.7%). After treatment the frequencies did not change significantly. In Haiti, we examined 84 hookworm eggs from 31 untreated subjects and 14 from five treated subjects and we did not identify any polymorphism at the SNP sites of interest. We recorded 19% and 63% of experimental failure for the samples from the pre- and post-treatment, respectively. In Panama, all 23 eggs analyzed from nine untreated subjects and 59 from 29 treated subjects were homozygous susceptible-type for all three positions. We recorded 71% and 24% of experimental failure for the samples from the pre- and post-treatment, respectively.

3.3.4. Drug Efficacy

The arithmetic means of the faecal egg count (FEC) per gram are presented in Table 3.2. The standard error of the mean obtained shows a high variability of the FEC in the treated and untreated populations.

The ERR estimates calculated for *A. lumbricoides*, *T. trichiura* and hookworms for each diagnostic test applied in Haiti, Kenya and Panama are summarized in Table 3.2. The ERR from the same country estimated using two different diagnostic methods are not directly comparable as the number of

samples tested (and therefore the hosts making up the ERR) were different. A direct comparison of the different diagnostic methods *per se* is beyond the scope of this paper and has been previously discussed elsewhere [29,30]. In Haiti where only McMaster was applied, the ERR for *A. lumbricoides* was the highest (99.9% (95% CI 99.5-100.0)) followed by hookworms (98.6% (95% CI 96.1-99.7)) and *T. trichiura* (49.7% (95% CI 0.0-88.4)). In Kenya the ERR estimates differed between the Kato-Katz and McMaster methods. The highest ERR was obtained for hookworms, with Kato-Katz at 89.95% (95% CI, 0.0-96.1%) and McMaster at 96.8% (95% CI, 92.5-99.2%). This was followed by *A. lumbricoides* with 97.3% (95% CI, 0.0-100.0%) and 80.3% (95% CI, 0.0-100.0%), respectively, and finally by *T. trichiura* with 86.8% (95% CI, 0.0-98.7%) and 10.1% (95% CI, 0.0-78%). The ERR estimate based on Kato-Katz was the one considered as the sample sizes available were much higher (n=104 and n=92 at pre- and post-treatment, respectively) than those for McMaster (n=24 and n=42 at pre- and post-treatment, respectively). In Panama, the ERR estimates for FLOTAC were the ones considered the number of samples analyzed was greater than those counted by Kato-Katz [19] and the number of samples analyzed was also greater [19]. In Panama, the highest ERR estimate was for *A. lumbricoides*, at 89.8% (95% CI, 75.8-97.3%), followed by *T. trichiura* with 65.1% (95% CI, 0-89.1%), and finally by hookworms with 47.8% (95% CI, 0-89.9%).

Table 3. 2. Faecal egg count reduction rates for *A. lumbricoides*, *T. trichiura* and hookworms. [1]FEC, faecal egg count, (egg per gram of stool). [2]ERR, egg reduction rate. The ERR estimates were obtained by dividing the difference between the arithmetic mean of the pre- and post-treatment FEC (at group level, ignoring individual variability) by the mean of the pre-treatment mean count (see text).[3]CI, confidence interval. The confidence intervals of the egg reduction rate were calculated using a bootstrap resampling method [31].[4]NA=Not applicable.

	Arithmetic mean FEC[1] at pre-treatment ± SE			Arithmetic mean of FEC at post treatment ± SE			ERR[2] (%) (95% CI[3])		
Country and Parasite	**McMaster**	**Kato-Katz**	**FLOTAC**	**McMaster**	**Kato-Katz**	**FLOTAC**	**McMaster**	**Kato-Katz**	**FLOTAC**
Haiti, n	353	[4]NA	NA	317	NA	NA			
Ascaris	370.9±248.9	NA	NA	0.2±20.2	NA	NA	99.9 (99.5-100)	NA	NA
Trichuris	48.4±13.1	NA	NA	29.1±12.8	NA	NA	49.7 (0-88.4)	NA	NA
HW[5]	179.8±50.0	NA	NA	2.9±21.3	NA	NA	98.6 (96.1-99.7)	NA	NA
Kenya, n	24	104	NA	42	92	NA			
Ascaris	2737.0±2707.1	330.5±284.3	NA	35.4±35.4	74.4±54.0	NA	97.3 (0-100)	80.3 (0-100)	NA
Trichuris	1650.0+1533.0	161.5±54.9	NA	96.6±69.9	145.2±63.5	NA	86.8 (0-98.7)	10.1 (0-78.1)	NA
HW	854.2±576.2	279.6±62.2	NA	3.57±2.6	6.3±3.0	NA	89.9 (0-96.1)	96.8 (92.5-99.2)	NA
Panama, n	NA	92	108	NA	65	223			
Ascaris	NA	8346.2±1952.4	1900.8±455.3	NA	3589.9±1927.4	171.9±70.9	NA	60.0 (0.0-95.1)	89.8 (75.8-97.3)
Trichuris	NA	280.8±170.1		NA	1.44±0.9	14.1±5.1	NA	99.5 (89.7-100)	65.1 (0-89.1)
HW	NA	196.9±253.7	72.6±24.8	NA	0.9±0.8	37.9±22.3	NA	99.8 (96.6-100)	47.8 (0-89.9)

3.3.5. Hardy-Weinberg Equilibrium

Guo's Exact Hardy-Weinberg test [27] showed that there was a significant departure from Hardy-Weinberg expectations recorded in *T. trichiura* collected in Haiti (p=0.0366) after treatment, and in Kenya before and after treatment (p<0.0001) for position 200 of the β-tubulin. This disequilibrium was characterized by a deficiency in the number of heterozygotes (Tables 3.3). The estimated frequency of a null allele at position 200 showed no evidence (χ^2= 3.69) that a null allele was responsible of the paucity of heterozygotes. For positions, 167 and 198, respectively, polymorphism was either not found, or the differences between the frequencies of heterozygotes pre- and post-treatment were not significant.

Table 3. 3. Observed (H_O) and expected (H_E) heterozygosity tested under HWE for SNP 200 in *T. trichiura*. [1]n=sample size.*Indicates a significant p-value (p<0·05); p-values are obtained from Guo's exact test.

	Pre- treatment				Post- treatment			
	n[1]	**H_O**	**H_E**	**p-value**	**n**	**H_O**	**H_E**	**p-value**
Haiti	65	0.25	0.24	1.00	38	0.26	0.43	0.0366*
Kenya	40	0.15	0.50	<0·0001	90	0.8	0.38	<0·0001

3.4. Discussion

One of the main findings of this study was the identification of a SNP at position 200 in *T. trichiura* samples collected in Haiti and Kenya at the pre-treatment collections. Thus, these findings suggest that the resistance-type allele (TAC_{200}) already existed in these populations prior to ABZ treatment. This result was consistent with a previous report in which the codon 200 polymorphism was identified in adult *T. trichiura* from Kenya in an ABZ-naïve population [17]. This SNP was also present in human filarial nematodes from Burkina Faso, in samples obtained from pre-treatment patients at a moderate allele frequency (26.2%) [32].

However, it must be taken into consideration that ABZ is a commonly used AH in endemic countries, and yearly school-based helminth control programmes relying on ABZ and MBZ are also common in countries where our studies were performed [33,34,35]. Indeed, in Kenya, six months prior to our study, children from the selected schools had been treated with ABZ in the context of the LF control programme. In Panama, at the baseline collection (nine months earlier), the codon 200 polymorphism was identified (data not shown), and in a previous study carried out in the same region, the same SNP had been detected in *T. trichiura* [17]. Finally, in Haiti, in the context of the LF control programme, ABZ is widely distributed yearly in combination with DEC at the community level, in surrounding communities [33]. So, it may be possible that some participants in Panama or Haiti from the studied populations had travelled to treated areas or could have been infected with eggs from family members who

had been previously treated with ABZ. Thus, it may be possible that parasites have been previously exposed to the drug and that some selection for the SNPs at codon position 200 had occurred prior to the beginning of MDA for STHs, or that in Kenya, selection at codon 200 may have developed following the previous rounds of ABZ treatment in the population.

Other findings were the identification of SNPs in *T. trichiura* at codon 167 in samples from Panama and at codon 198 in samples from Haiti and Kenya, prior to treatment. The identification of the same resistance-type allele at codon 198, in populations that are geographically separated suggests that this allele could be common in *T. trichiura* populations, even prior to ABZ treatment. However, the frequency of the resistance-type allele at codon 198, and the identification of the resistance-type allele at codon 167 in only one country suggest that these polymorphisms are less common than the codon 200 polymorphism. In the veterinary parasitic nematode *Haemonchus contortus*, it has been confirmed that the codon 200 polymorphism, which causes BZ resistance, is the predominant SNP associated with resistance, compared to the codon 167 polymorphism [13], or the codon 198 polymorphism [12,15]. In addition, it was also interesting to note that the codon 167 polymorphism was always found alone and never associated with the SNPs at positions 198 and 200. This is consistent with observations made in *H. contortus* suggesting that the polymorphism at position 167 (TAC) does not occur in the same allele as the polymorphism at position 200 (TAC) or 198 (GCA) [12,15].

The post-treatment examination of the resistance-type allele frequencies of *T. trichiura* collected in the three different geographical areas suggests that ABZ may be selectively eliminating worms carrying susceptible-type alleles and allowing worms with the resistance-type alleles to survive treatment. Indeed, in Haiti and Kenya one round of treatment significantly increased the frequency of the homozygous resistance-type at codon position 200 (TAC/TAC_{200}) and some heterozygote eggs (TTC/TAC_{200}) were also found after treatment. It was also found that the codon 198 polymorphism persisted after one treatment but at lower frequency compared to the codon 200 polymorphism. Thus, our results suggest that ABZ treatment could select for parasites harbouring the codon 200 (TAC) polymorphism. We tested whether the genotype frequencies of the β-tubulin gene at codon 200 obtained before and after treatment were in HWE. In Haiti, the increase of the homozygous resistance-type after treatment, for codon position 200, correlated with a departure from HWE illustrated by a paucity of heterozygotes and an excess of homozygotes. In Kenya, the β-tubulin at position 200 in *T. trichiura* was not in HWE before treatment as there was an excess of homozygotes. Different hypotheses may explain the HWE inbalance in genotype frequencies; one possible cause could be selection acting on the parasite populations.

The above data strongly suggest that the SNP at β-tubulin codon 200 may be responsible for the intermediate efficacy of ABZ against *T. trichiura*, where the ERR ranged from 10.1% (Kenya) to 65% (Panama). A poor efficacy of ABZ against *T. trichiura* has been repeatedly observed across a number of different

studies [36]. In addition, it is not uncommon to find considerable variation of ERR estimates (ranging from 0 to 90%) in the literature [37], which, in addition to being explained by the use of different diagnostic methods (see above), is in agreement with the high resistance-type allele frequencies observed at loci 200 and 198 in Haiti and Kenya. If drug susceptibility is determined (in part) by these alleles, then AH resistance might develop relatively quickly in locations such as these. Resistance to BZ drugs is usually recessive [11] so high allele frequencies are required to generate the phenotypic diversity that leads to the poor drug efficacy that has been recorded.

Another finding of the study was the identification of the resistance-type SNP (based on comparison with studies in some veterinary nematodes), at codon position 167 in *A. lumbricoides* before and after treatment. In *A. lumbricoides* collected in Haiti, Kenya and Panama, the allele containing TAC at codon 167 was identified as homozygous at high frequency prior to ABZ treatment. The post-treatment genotype frequencies indicated that ABZ treatment did not change the frequency of the homozygous, resistance-type, codon 167 in Kenya and Panama, where sample sizes were similar, and heterozygotes remained in the population surviving treatment. It was also interesting to note that similar findings have been published for the veterinary parasitic nematode, *Teladorsagia circumcincta* [13]. In this case, it was demonstrated that BZ treatment did not change the frequency of the homozygous, resistance-associated codon 167 or that of heterozygotes. In a recent study, it was also found that *H. contortus* heterozygous at codon 167 were associated with susceptible phenotypes [15]. In

contrast, in Haiti where the sample sizes were highly different between the pre- and post-treatment groups, we found that the resistance-type genotype frequency of *A. lumbricoides* at codon position 167 significantly increased after treatment, but the small sample size at the post-treatment collection made it difficult to draw clear conclusions.

The genetic data from *A. lumbricoides* suggest that the polymorphism at codon position 167 is common and may be naturally present. However, ERR estimates indicate that treatment was successful according to WHO standardized thresholds [38]. This implies that in *A. lumbricoides*, the mutation at codon position 167 may not have an impact on drug efficacy. In contrast, in intestinal trichostrongylid nematodes of livestock (e.g., *H. contortus*), the SNP 167 is rarely encountered but the homozygous allele containing TAC does confer BZ resistance in some species [39,40]. It is also common in these species to have SNP associations between codons 167 and 200 that could confer high levels of BZ resistance [15].

It was interesting to find that in *A. lumbricoides*, all eggs genotyped were susceptible-types at codon positions 198 and 200. It is possible that should polymorphisms be found at codons 200 or 198 in *A. lumbricoides*, these polymorphisms may change the sensitivity of the parasite to the drug as has been described in veterinary parasites [12,15]. It will be informative to investigate the β-tubulin gene of *A. lumbricoides* in areas where MDA has been implemented to determine whether the position 200 is ever polymorphic and whether it correlates with drug response.

Finally, the β-tubulin analysis in hookworms, at codon 200, 167 and 198, revealed the presence of a SNP at codon 200 in samples collected in Kenya at pre- and post-treatment. However, the frequency was very low and the ERR indicated a value consistent with ABZ treatment being successful. In other studies in which a reduced drug efficacy was detected polymorphism at codons 200 and 167 was not detected [41,42].

The main limitation of the present study was the non homogeneity of the study designs in Haiti, Kenya and Panama. Indeed, the samples and data on response to treatment were obtained as part of different collaborations with ongoing treatment protocols rather than being designed specifically for our study. As such it was not possible to standardize the methods used for egg count estimations and exact time samples were taken after treatment. Furthermore, the studied populations were different in the three studies. In Panama and Kenya it was restricted to pre-school and school age children whereas in Haiti, adults and children were sampled. Furthermore, three diagnostic methods have been applied to quantify STHs. Resulting drug efficacies were variable within the same country, depending on the techniques and sample sizes. In another study, the McMaster and Kato-Katz gave similar results for the ERR [25]. It is important to mention that the sample sizes were different for the two diagnostic tests when performed in the same country. Our goal was not to compare test sensitivity for quantifying egg counts. The results reported here should be viewed as those arising from three different field treatment studies in which we used procedures to assess genotype and have compared the genotypes with assessments of ERR for

each of the three separate treatment studies, observed with different methods and sampling schedules. As such the ERR results between the studies should not be compared; what was relevant for our investigations was the assessment of ERR by one or more conventional parasitological methods with the genotype determination for samples from the same communities.

The other limitation of the study was the low sample size of individual eggs genotyped due to a high percentage of PCR failure. This was caused by the difficulty in amplifying the DNA of individual eggs, possibly due to the presence of inhibitors from faeces that could prevent the DNA extraction [43]. Also, the low DNA concentration may have affected the success of the DNA amplification and pyrosequencing. In *T. trichiura* eggs from Panama, we recorded more DNA amplification failure for positions 198-200 than for position 167. One of the primers used to amplify the sequence around the codons 198 and 200 was in part in an intron. Polymorphism in this intron may have resulted in poor amplification in some samples. However, the samples that were successfully genotyped allowed identification of SNPs in *T. trichiura* which may be associated with the ability of worms to survive ABZ treatment. These results stress the importance of implementing, as integral part of MDA programmes, regular monitoring of drug efficacy and associated parasite genetics for prompt detection of drug resistance.

In this study, we estimated the ERR at the group level; it is a more accurate estimation of overall ERR because it can include negative individual values. The estimation of the ERR on a group basis by bootstrapping gave a range

of [0-89%] in hookworms, whereas when estimated by Halpenny and colleagues [19] on individual basis the ERR was 89%.

Likewise, there is an urgent need to consider control strategies that will maintain a low resistance-type SNP prevalence and a high drug efficacy. Strategies have been described to maintain a high level of STH control while also delaying the possible development of drug resistance [7]. Drug combination has been used to combat the problem of AH resistance in veterinary parasites and human parasites [44,45]. The pros and cons of various combinations should be carefully considered so that the chosen combination will have both a positive impact on drug efficacy and on a reduction of genetic selection for resistance.

3.4.1. Conclusion and future directions

Comparable data on pre- and post-treatment SNP frequencies were obtained at the different study sites, despite differences in the study designs between sites. However, in the context of monitoring and surveillance of STH control programmes, it will be important to conduct multiple studies according to standardized multi-centre protocols in order to allow drug efficacies to be compared.

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CONNECTING STATEMENT III

In chapter III, we identified at low frequency a polymorphism at codon position 200 in hookworms collected in Kenya before and after treatment. However, drug efficacy against hookworms was high showing that there was no phenotypic evidence of resistance. We also identified in *A. lumbricoides* a polymorphism at codon position 167 in both pre- and post-treatment samples collected in the three field sites, while drug efficacy remained high. Thus, this polymorphism did not seem to affect drug efficacy. Finally, we identified a SNP at codon 198 in *T. trichiura* collected before and after one round of ABZ treatment in Haiti and at codon 200 in Haiti and Kenya. The polymorphism significantly increased after one round of treatment and the drug response against this parasite was low in Kenya and moderate in Haiti. To confirm the genotypic selection observed we carried out a longitudinal study in Haiti to assess the SNPs in *T. trichiura* before treatment, and two weeks, two months and one year after treatment. Subjects infected with *T. trichiura* were classified into three groups of responders: poor, intermediate and good. The resistance genotypes were determined for each group of responders before and after one treatment. It was also of interest to assess drug efficacy at the individual level in subjects infected with *A. lumbricoides* and hookworms. All results are presented in the next chapter.

CHAPTER FOUR

Longitudinal studies on soil-transmitted helminths in five Haitian endemic communities and association between the β-tubulin polymorphism in *Trichuris trichiura* and response to albendazole

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Manuscript in preparation

Abstract

The STHs *Ascaris lumbricoides*, *Trichuris trichiura* and the two hookworms *Necator americanus* and *Ancylostoma duodenale* are the causative agents of soil-transmitted helminthiases. To control these harmful parasites, ABZ is one of the drugs periodically distributed through MDA programs at the community level in endemic countries. It is reported in the literature that a single dose of ABZ shows high activity against *A. lumbricoides,* and moderate and low activity against hookworms and *T. trichiura*, respectively. This low drug activity against *T. trichiura* suggests that the parasite may contain resistance codons in the β-tubulin gene, which limits the drugs activity against a proportion of the parasitic population. We have carried out a 14-month longitudinal study between 2009 and 2010 in five endemic communities for STHs in Haiti. Stool samples were collected at three different times in 2009, once before treatment and then two weeks, and two months after a single treatment. In 2010, two collections were performed, one prior to treatment and two weeks after treatment. The McMaster technique was used to estimate the feacal egg count at pre- and post-treatment and the ERR was calculated at a group and individual level for *A. lumbricoides* and hookworms and only at an individual level for *T. trichiura.* Infected subjects with *T. trichiura* were classified into three response groups (good, intermediate and poor), and the frequency of the polymorphisms at codons 167, 198 and 200 was assessed for each group. For the other two parasites, the polymorphism frequency was also assessed. Furthermore, we also determined in this study the dynamics of

infection (prevalence, intensity, and risk factors) for the three parasites. For *T. trichiura*, we identified a significant increase in resistance genotypes at codons 198 and 200 after one treatment in the intermediate and poor responder groups. In these same groups, we noticed a persistence of the parasites carrying a resistance allele two months after treatment. One year post-treatment we still identified 16.7% and 83.3% of the resistance alleles at positions 198 and 200, respectively, in subjects classified as being in the poor responder group at baseline. In hookworms, we did not identify any polymorphisms and in *A. lumbricoides*, we identified a polymorphism at codon 167, however, drug efficacy remained high. This study confirmed that selection of resistance alleles in *T. trichiura* occurred and also provided phenotypic evidence of apparent resistance. In addition, this epidemiological study revealed the major risk factors for infection with *A. lumbricoides, T. trichiura,* and hookworms.

4.1. Introduction

The human STHs, *Ascaris lumbricoides*, *Trichuris trichiura* and the two hookworms *Necator americanus* and *Ancylostoma duodenale* are gastrointestinal nematodes. Their distribution, prevalence, intensity and clinical manifestations contribute to public health problems in the tropical countries of the world [1-3]. The main strategy to control STHs consists of MDA of ABZ or MBZ [4]. In Haiti, especially in Léôgane, in the context of the LF program, over the past decade the use of ABZ in combination with diethylcarbamazine (DEC) has been central to the control of intestinal parasites through community-based treatment programs [5,6]. In veterinary nematodes, it is well known that the frequent use of the BZ AHs can exert selection pressure on the β-tubulin gene that causes resistance [7-9]. The possibility of selection for drug resistance in STHs, notably in hookworms and *T. trichiura* has been reported in both naive and treated communities in Haiti, Kenya and Panama [10] (Chapter 3). In a previous study, we have highlighted the importance of assessing the frequency of SNPs at codon positions 198 and 200 in the β-tubulin gene of *T. trichiura* before and after treatment and its positive association with the intermediate-poor efficacy observed with ABZ treatment.

In the present work, we performed a longitudinal study using epidemiological and molecular analysis. The epidemiological analysis was carried out in Haiti and consisted of a 14-month longitudinal study in which prevalence, egg intensity, drug efficacy and risk factors of infection with each STH were determined, and STH eggs from infected participants were collected. The molecular analysis of the collected eggs was then done at McGill University,

Canada and we assessed the frequency of SNPs in the β-tubulin gene, previously shown to be associated with BZ resistance in veterinary nematodes, for *A. lumbricoides* and hookworms. We also found that the genetic changes in the β-tubulin gene and egg count reductions provide strong genotypic and phenotypic evidence of emerging ABZ selection for resistance in *T. trichiura.*

4.2. Material and method

4.2.1. Study area and population

The longitudinal study was conducted between April 2009 and August 2010 in five Haitian communities endemic for STHs. The communities were selected based on the fact that they had never been involved in previous MDA programs, although individual treatments were likely to have taken place. Stool samples were collected from volunteer children and adults meeting the inclusion criteria (older than 2 years old). Four communities were located in the municipality of Grand-Goave (18°25'44"N 72°46'14"W) (Montsembé, Grand-Goave, Papatambre and Teno) in the West part of Haiti and one (Kavette) in the municipality of Cayes-Jacmel (16°16'0"N 72°40'0"W) in the Southeast part (Figure 4.1). Also, four communities (Montsembé, Papatambre, Teno and Kavette) included in the study were rural and one was urban (Grand Goave).

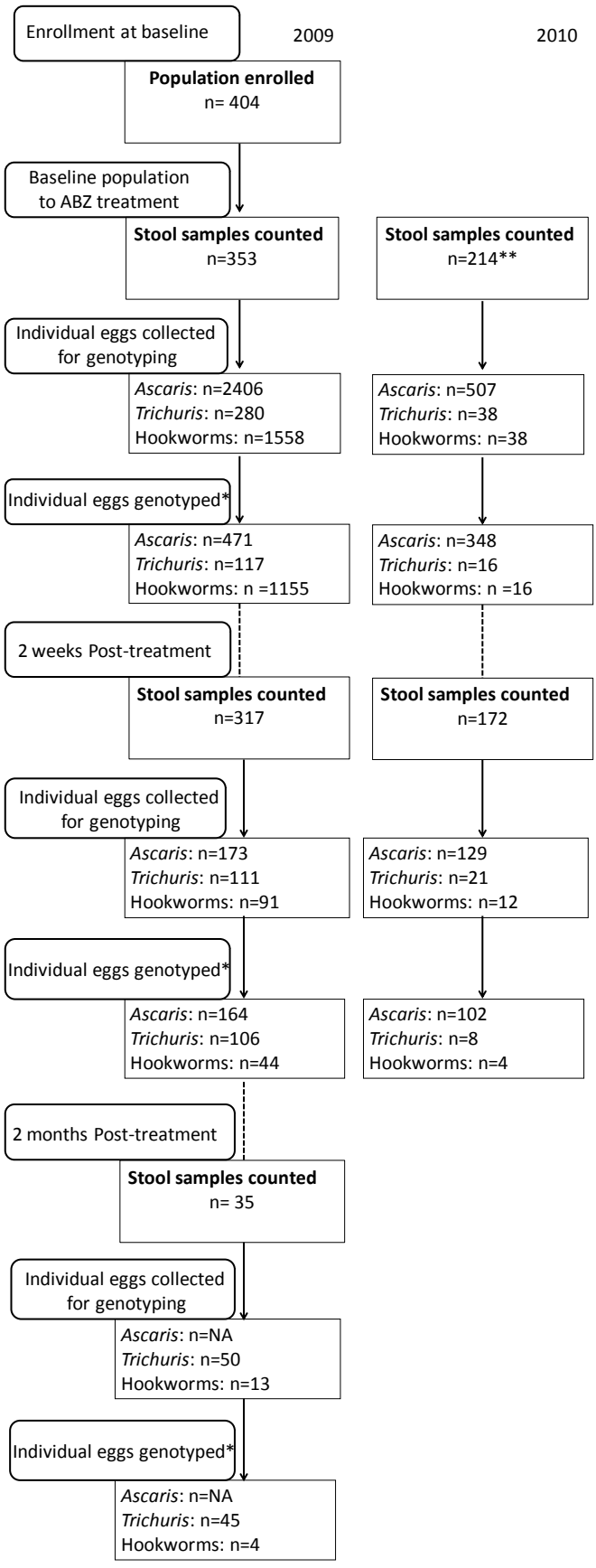
4.2.2. Field procedures

Recruited participants were identified through a questionnaire, and containers were distributed to each of them. Participants were asked to fill them with a fresh stool sample, which were subsequently collected the following morning from each community. Over the 14-month longitudinal study, a total of five stool samples were collected and two ABZ treatments were administered. The baseline collection was obtained in April 2009 (n=353), after that, ABZ (400 mg) in combination with DEC (6 mg/kg) was distributed to everyone in each community in the context of an integrated program to control LF and intestinal parasites. Follow-up feacal samples were collected two weeks after drug treatment (n=317) in all communities and again 2 months later (n=35) in all communities except Kavette because of transportation issues. In June 2010, approximately one year after the first treatment, a follow-up collection was undertaken in the same communities (n=214) that also served as a pre-treatment baseline. Thus, the participants from the 2009 study were expected to be enrolled in the follow-up study, however six months before, in January 2010, Haiti was hit by a 7.0-magnitude earthquake. This tragedy had a huge impact on our study. First of all, in the urban community (Grand-Goave), located only 18 km from the epicenter, most of the infrastructure (including houses) collapsed and so people were relocated to shelters. In this community, except for two people, we lost track of the rest of the participants enrolled in the 2009 study. To balance the sample size, new people from this same community, and who had received the previous year's MDA, were included.

In addition, the loss of vehicles usually used for the different activities linked to the LF program, caused a major logistic issue for transportation. This issue prevented us from going to the rural communities on specific days (market day) or times (after school) to reach the maximum number of participants for collecting samples in the second year.

All communities were treated for the second time with ABZ, and two weeks later stool samples were collected from the same individuals (n=173). The drug was a donation from GlaxoSmithKline (GSK). Participants in the study were not observed during drug administration. Thus, treatment compliance was evaluated post-treatment through a questionnaire and we relied on self-reported information from each participant, and on pre- and post-treatment egg counts. Samples from participants who were not treated with ABZ were not included in the analysis. A detailed survey flow diagram is shown in Figure 4.1.

Figure 4. 1. Flow diagram of the participation, sample submission and analysis conducted during the 14-month longitudinal study on soil-transmitted helminths. *Caused by experimental failure, the number of eggs genotyped is lower than the number of eggs counted. **In the urban community, in 2010 new people were included in the study to replace participants from the 2009 collection who could not be tracked (see text). NA: Not applicable.The dashed lines mean that the connection between the two boxes is not direct.


Enrollment at baseline
2009
2010
Population enrolled
n= 404
Baseline population to ABZ treatment
Stool samples counted
n=353
Stool samples counted
n=214**
Individual eggs collected for genotyping
Ascaris: n=2406
Trichuris: n=280
Hookworms: n=1558
Ascaris: n=507
Trichuris: n=38
Hookworms: n=38
Individual eggs genotyped*
Ascaris: n=471
Trichuris: n=117
Hookworms: n =1155
Ascaris: n=348
Trichuris: n=16
Hookworms: n =16
2 weeks Post-treatment
Stool samples counted
n=317
Stool samples counted
n=172
Individual eggs collected for genotyping
Ascaris: n=173
Trichuris: n=111
Hookworms: n=91
Ascaris: n=129
Trichuris: n=21
Hookworms: n=12
Individual eggs genotyped*
Ascaris: n=164
Trichuris: n=106
Hookworms: n=44
Ascaris: n=102
Trichuris: n=8
Hookworms: n=4
2 months Post-treatment
Stool samples counted
n= 35
Individual eggs collected for genotyping
Ascaris: n=NA
Trichuris: n=50
Hookworms: n=13
Individual eggs genotyped*
Ascaris: n=NA
Trichuris: n=45
Hookworms: n=4

4.2.3. Laboratory procedures

Stool samples were transferred to the Hôpital Sainte-Croix laboratory in Léôgane, Haiti. A modified McMaster egg counting technique was applied on samples as described in Chapter 3. One gram of feacal material was added to water and the solution was stirred until it was completely broken apart. The mixture was then poured through surgical gauze into a centrifuge tube. After centrifugation for 10 min at 15,000 rpm, the supernatant was poured off and the tube containing the sediment was filled with a saturated sucrose solution and gently stirred. After 10 min, an aliquot of the fluid from the upper phase of the solution was transferred into each McMaster chamber compartment. The eggs were counted in both chambers using a low power objective (×10). The number of eggs per gram (epg) of feaces was obtained by multiplying the total number of eggs counted in the two chambers by 50. The remaining stool samples were put into anaerobic containers as described previously [11] and eggs from positive subjects were recovered using a saturated sucrose solution subjected to centrifugation as described above, and then preserved and stored in 70% alcohol until molecular analysis could be performed (See Figure 4.1).

4.2.4. Evaluation of the risk of infection with STHs

In addition to stool collection, individual (age, sex, and occupation) and household data (latrine access, density of the household, and household habits; e.g. meal at a table or on the floor) were recorded at baseline in 2009 and 2010 for each participant to evaluate factors that could influence the risk of infection to STHs. Anthropometric data was also collected on each individual. The height-for-

age Z-score (HAZ) for children (2 to 19 years old) and the body mass index for adults (≥ 20 years old) were assessed to determine the prevalence of stunting and malnutrition associated with a low BMI in infected (2009: n=115, 2010: n=21) and non-infected (2009: n=238, 2010: n=193) individuals. Height and weight of participating adults and children were measured using a portable stadiometer (Seca 214, Birmingham UK) and scale (Seca 761, Birmingham UK). Child (2009: n=189, 2010: n=121) HAZ scores were calculated using the WHO growth reference standards, and the WHO Anthro Plus version 1.0.4 software [12]. Children were categorized as stunted if their HAZ was ≤-2SD. The adult BMI (2009: n=164, 2010: n= 93) was calculated as the ratio between the weight in kilograms and the square of the height in meters, and adults were classified as underweight if they had a BMI <18.5 [13].

4.2.5. Ethics statement

Ethics approval was obtained from the Institutional Review Board of the Centers for Disease Control and Prevention, Atlanta, Georgia, USA (Dr. Patrick Lammie), and the Ethics Committee of the Hôpital Ste. Croix, Haiti and included the collection of stool samples, examination of stool samples for helminth eggs and DNA analysis of helminth eggs. Informed consent was obtained from all adult participants and from parents or legal guardians of all minors. A parent or legal guardian gave consent in every case in which a child participated. Based on past experience, it was likely that some people in the communities would not be able to read. A waiver of written informed consent was requested and approved on the basis that the research presented no more than a minimal risk of harm to the

subjects and involved no procedures for which written consent is normally required outside the research context in this setting. The use of oral consent was previously approved by the IRB. Subjects were offered a written copy of the IRB approved consent form. The contents of the approved consent form were explained to each household, following which verbal consent was obtained. The reader of the consent form and a witness signed a copy of the form to indicate the subject's agreement.

4.2.6. Molecular analysis of the β-tubulin of STH eggs collected

All eggs previously collected in the field were preserved in 70% alcohol and then separated by species as single eggs or pools of 10, 50, 100 or 500 eggs, under a microscope at McGill University. DNA from each individual or pool of eggs was subsequently extracted as described (Chapter 3). After DNA extraction, all samples were subjected to PCR to amplify small fragments surrounding the SNPs at codon positions 167, 198, and 200 of the β-tubulin genes of *A. lumbricoides* (FJ501301.1, GenBank acc. number), *T. trichiura* (AF034219.1 GenBank acc. number) and hookworms (EF392851.1 GenBank acc. number). All primer sequences and PCR conditions used were as previously described (Chapter 3). The codon positions 167, 198, and 200 of all amplified PCR products were then subjected to genotyping by pyrosequencing.

4.2.7. Statistical analysis

All continuous data obtained to characterize the interviewed population was reported as the mean ± SD and calculated using IBM SPSS Statistics version 20.0, Released 2011 (IBM SPSS Armonk, NY: IBM Corp).

The primary outcomes measured were prevalence, egg intensity, estimated as the epg, and the ERR. Binomial confidence limits (95%) for prevalence were assessed using IBM SPSS Statistics version 20.0, Released 2011 (IBM SPSS Armonk, NY: IBM Corp). The epg calculated at the pre- and post-treatment collections for each parasite were compared using the Wilcoxon test due to the non-normal distribution of the data, within Graphpad Prism (GraphPad software, San Diego, CA, USA). The ERR was calculated to assess the drug efficacy after each ABZ treatment. The ERR was calculated at the group level as the ratio of the difference between the arithmetic mean of the pre- and post-treatment faecal egg count (FEC to the pre-treatment arithmetic mean, expressed as a percentage, ignoring the individual variations. Confidence intervals (CIs) for the ERR at group level were determined using a bootstrap resampling method (with replacement) over 10,000 replicates in R (version 2.15.0, Vienna Austria, http://www.R-project.org). The ERR was also calculated at the individual level as described [14]. In individual subjects carrying *T. trichiura*, according to the ERR, the response to ABZ was classified into 3 categories: good, intermediate and poor.

The secondary outcomes measured were the risk factors of infection with *A. lumbricoides*; *T. trichiura* and hookworms adjusting for individual and household covariates with a multiple logistic regression model. To take into

account the clustered nature of the data, we used the method of Generalized Estimating Equations (GEE) [15]. This method takes into consideration the possible correlation between subjects from the same community. We assumed that the correlation between observations made on subjects in the same community did not have any particular structure (unstructured). The GENMOD procedure in SAS, version 9.2 (SAS Institute Inc., Cary, NC) was used.

Genotype frequency of SNPs at positions 167, 198 and 200 in *A. lumbricoides*, *T. trichiura* and hookworms obtained at the pre-treatment collections were compared with the genotype frequency of the same SNPs obtained at the post-treatment collections using Fisher's exact test within GraphPad Prism (GraphPad software, San Diego, CA, USA).

4.3. Results

4.3.1. Characteristic of the baseline

In our longitudinal study, a total of 353 and 214 individuals from 66 households were sampled in 2009 and 2010, respectively. Their ages ranged from 2 to 81 years with a mean age of 26.0 ± 19.9 and 25.0 ± 19.5 in 2009 and 2010, respectively. The most common occupation of participants was student (55% for both years), followed by farmer, accounting for around 20% of the participants for both years. Information collected on household features revealed that around half of the households interviewed had access to latrines (51.6% in 2009 and 53.4% in 2010) and the density of their households ranged from 0.5 to 8 people per room

with a mean density of 2.6 ± 1.3 for both years. In addition, among the interviewed households, slightly more than half had their meal on a table (instead of having it on the floor in 2009), and that proportion increased (65.1%) in 2010 (Table 4.1).

The anthropometric data revealed a low proportion of stunted children, (16.4% in 2009 and 16.5% in 2010), and the HAZ score ranged from -3.3 to 6.3 with a mean of -0.5 ± 1.9 and -0.5 ± 2.0 in 2009 and 2010, respectively (Table 4.1). We did not find any significant difference (p values = 0.2005 for 2009 and 2010) in stunting between the infected and non-infected children (data not shown). In the adult population, the BMI ranged from 13.7 to 34.1 in 2009 with a mean of 21.1 ± 3.4 and from 14.5 to 32.4 in 2010 with a mean of 21.4 ± 3.5 (Table 4.1). The prevalence of underweight individuals in adult populations (BMI between 18.5 and 16.0) was 14.6% in 2009 and 18.3% in 2010, and 4.3% of adults were severely underweight (BMI ≤ 16) in 2009 and 3.2 % in 2010. As observed for children, there was no significant difference (p-value=0.1861 for 2009 data and p-value=0.5938 for 2010 data) in the prevalence of underweight or severely underweight adults, whether infected or not (data not shown).

Table 4. 1. Characteristics of the baseline population included in the study in 2009 and 2010. [1]The new participants from the urban community were included in the total number of participants in 2010. [2]In 2009 and 2010 the minimum age of the participants was 2 years old and the maximum was 80 in 2009 and 81 in 2010. [3] Children and teens were ages from 2 to 19 years old. [4]Adults were 20 years of age. [5]Other occupations included: tailor, builder, unemployed, retired, fisher, and garage mechanic. [6]There was a minimum of 0.5 and a maximum of 8 people per room in both years. [7]Stunting was defined as a height for age z-score (HAZ) value less than -2. [8]An individual with a BMI less than 18.5 was considered underweight.

	Baseline 2009	**Baseline 2010[1]**
Subject, n (%)	353 (100)	214 (100)
Age at enrolment, mean ± SD[2]	26.0±19.9	25.0±19.5
Children[3], n (%)	189 (53.5)	121 (56.5)
Adult, [4] n (%)	164 (46.5)	93 (43.5)
Sex, n (%)		
Male	144 (40.8)	80 (37.2)
Female	209 (59.2)	135 (62.8)
Occupation, n (%)		
Street vendor	56 (15.9)	38 (17.7)
Farmer	71 (20.1)	44 (20.5)
Preschooler/primary/secondary+	194 (55)	120 (55.8)
Other[5]	32 (9.1)	13 (6.0)
Household, n	66	66
People per room[6], mean ±SD	2.6±1.3	2.7 ±1.3
Access to latrines, n (%)		
Yes	182 (51.6)	115 (53.4)
No	171 (48.4)	100 (46.6)
Eat on table		
Yes	197 (55.8)	140 (65.1)
No	156 (44.2)	75 (34.9)
Height for age		
Z-score, mean±SD	-0.5±1.9	-0.5±2.0
Stunting[7], n (%)	31 (16.4)	20 (16.5)
BMI[8], mean ±SD	21.1±3.4	21.4±3.5

4.3.2. Prevalence and intensity

Overall 353 individuals submitted stool samples for the baseline collection in 2009, and the FEC was obtained using the McMaster technique. The prevalence of hookworms was 19.5% (95% CI, 15.4 %-23.7 %); *T. trichiura* and *A. lumbricoides* were diagnosed in 10.8% (95% CI, 9.6%-14.0%) and 8.5% (95% CI, 7.5 %-11.4%) of the participants, respectively (Figure 2). However, the highest intensity was detected for *A. lumbricoides* (5,000 epg), then hookworms (1,200 epg), and finally *T. trichiura* (410 epg). After one round of treatment with ABZ, egg intensity significantly decreased for the three parasites (p=0.0313). Indeed, the intensity decreased by around 4-fold and 60-fold after treatment of *A. lumbricoides* and hookworms, respectively, whereas for *T. trichiura* the reduction was less than 2-fold after the same treatment (Figure 4-2 and 4-3).

By 2 months post-treatment, the prevalence of infection with hookworms reached the baseline level of 20% (95% CI, 6.7%- 33.2 %), whereas for *T. trichiura* the prevalence remained low (4.8%) and no individual was diagnosed with *A. lumbricoides* (Figure 4-2).

One year post-treatment in 2010, the prevalence of infection remained low and ranged from 2.8% (95% CI, 2.3-5.2%) for *T. trichiura* and hookworms, to 4.2% (3.8-7.2%) for *A. lumbricoides.* However, the intensity of infection was close to the levels observed at the pre-treatment collection in 2009 (Figure 4.3). After a second round of ABZ treatment a statistically significant decrease in intensity (p-value=0.0313) was observed, with the reduction being greater than after the first treatment. The prevalence was also very low for the three STHs after

the second treatment (Figure 4-2): 0.6% (95% CI, 0.5-2.4%) for *A. lumbricoides*, 3.5% (95% CI, 2.6-6.1%) for *T trichiura* and 1.2% (95% CI, 0.5-2.4%) for hookworms.

Figure 4. 2. Prevalence, with 95% confidence intervals, of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms among selected Haitian communities in 2009 and 2010 before and after albendazole treatment. Prevalence of *Ascaris* (A), *Trichuris* (B) and hookworms (C) after five stool collections. A single dose of albendazole (400 mg) was administered after the baseline (BL) collections in 2009 and 2010.

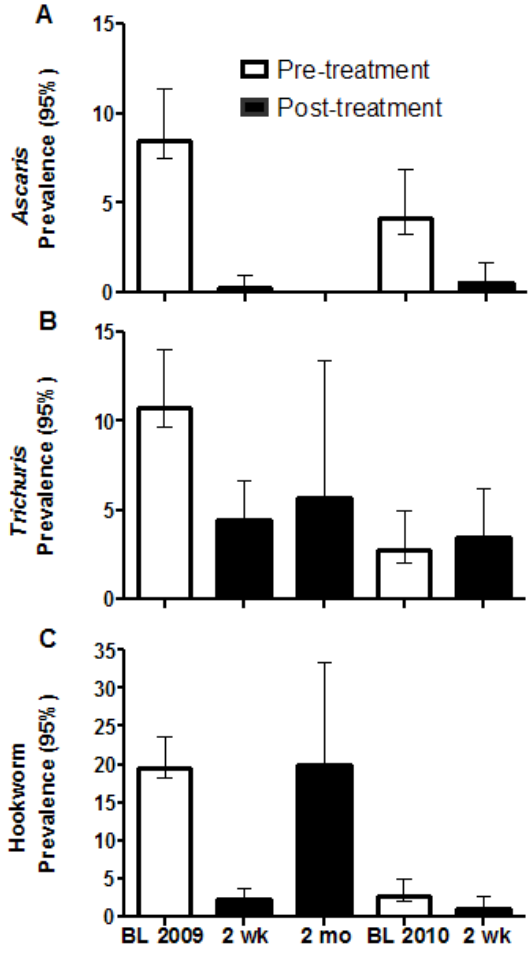
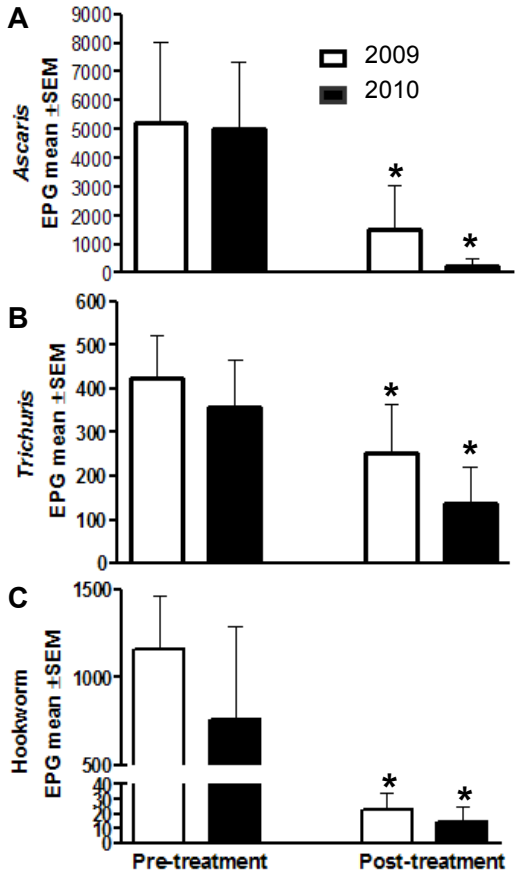


Figure 4. 3. Intensity of infection with *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms at the pre- and post-treatment collection in 2010 and 2010. White bars indicate the collections in 2009, black bars indicate the collections in 2010. In both years, collections were performed before and after albendazole treatment. The intensity of infection with *Ascaris*, *Trichuris* and hookworms was determined by the number of eggs counted in one gram of feacal material, (epg). The epg was assessed by McMaster technique. *Indicates a significant decrease in epg after treatment (p=0.0313). P-values were obtained by Wilcoxon test.



4.3.3. Risk factors of infection with *A. lumbricoides*, *T. trichiura* and hookworms

Results of the logistic regression models fitted for STHs are displayed in Tables 4.2 and 4.3. Multivariate models showed that at baseline in 2009, working as a farmer (*A. lumbricoides*: OR, 3.6; 95% CI, 1.0-12.5; hookworms: OR, 2.6; 95% CI, 1.1-5.8) increased the risk of being infected with these parasites. In addition, being male (OR, 1.6; 95% CI, 1.1-2.4) and having a higher household density were also risk factors for infection with *T. trichiura* (Table 4.2).

A similar pattern was observed in 2010, one year after the first treatment, in that we found a significant association between being male and being infected with *T. trichiura* (OR, 2.3; 95% CI, 1.3-4.6). In contrast, being a farmer seemed to reduce the prevalence of infection with *T. trichiura* (OR, 0.2; 95% CI, 0.0-0.8), compared to other occupations (Table 4.3).

Data on participants from the urban community was not included in the analysis of risk factors..

Table 4. 2. Multiple regression models to assess the risk of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm infection in the 314 participants from the rural[1] areas before treatment in 2009. [1]Participants from the urban community (n=39) were not include in the model. [2]Includes street vendors and the group "others" (tailor, builder, fisher, unemployed, retired, fisher and garage mechanic). Variables in parenthesis (versus variable) indicate the baseline comparison, and figures in bold indicate significant probability. CI, confidence interval. OR, odd ratio.

Variable	*Ascaris*		*Trichuris*		Hookworm	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Individual						
Age	0.4 (0.1-1.7)	0.073	1.0 (0-0.1)	0.524	1.0 (1-1)	0.906
Male (vs female)	0.9 (0.9-1.3)	0.567	**1.6 (1.1-2.4)**	**0.0212**	1.1 (0.8-1.5)	0.440
Farmer (vs student)	**3.6 (1.0-12.5)**	**0.042**	0.6 (0.2-2.3)	0.504	1.6 (1.5-4.1)	0.282
Farmer (vs other[2])	3.0 (0.7-11.9)	0.118	0.4 (0.1-1.1)	0.072	**2.6 (1.1-5.8)**	**0.025**
Household						
Do not eat at table (vs do eat at table)	1.1 (0.6-1.4	0.686	0.7 (0.1-0.2)	0.082	0.9 (0.7-1.2)	0.574
Latrine access (vs no access)	0.9 (0.6-1.3)	0.567	1.0 (0.6-1.7)	0.854	1.0 (0.7-1.3)	0.827
# people per room	1.0 (0.8-1.4)	0.898	**1.9 (1.4-2.5)**	**<0.001**	0.9 (0..6-1.2)	0.347

Table 4. 3. Multiple regression models to assess the risk of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm infection in the 184 participants from the rural areas[2] before treatment in 2010. [1]Participants from the urban community (n=39) were not included in the model.[2]Includes street vendors and the group "others" (tailor, builder, fisher, unemployed, retired, fisher and garage mechanic). Variables in parenthesis (vs variable) indicate the baseline comparison and figures in bold indicate significant probability. CI, confidence interval. OR, odd ratio. A single dose of ABZ (400 mg) was delivered in all communities in 2009 after the baseline collection.

Variable	*Trichuris*		Hookworm	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Individual				
Age	1.0 (0.9-1.0)	0.984	1.0 (0.9-1.1)	0.847
Male (vs female)	**2.5 (1.3-4.6)**	**0.003**	1.0 (0.5-2.2)	0.997
Farmer (vs student)	0.9 (0.0-1.4)	0.976	2.7 (0.2-35.7)	0.451
Farmer (vs other[2])	**0.2 (0.0-0.8)**	**0.023**	1.8 (0.2-13.9)	0.552
Household				
Do not eat on table (vs do eat at table)	1.2 (0.4-3.3)	0.720	2.1 (0.7-6)	0.166
Latrine access (vs no access)	2.1 (0.8-5.3)	0.117	0.7 (0.4-2.2)	0.903
# people per room	**2.5 (1.0-6.7)**	**0.054**	0.8 (1.3-1.5)	0.656

4.3.4. SNP frequencies and drug efficacy in hookworms and *A. lumbricoides*

In hookworms, we did not detect polymorphisms at codon positions 167, 198 or 200 in any individual egg or egg pools analyzed from the 2009 and 2010 collections. All eggs genotyped were homozygote susceptible types (TTC/TTC) at codon positions 167 and 200, and GAA/GAA at codon position 198. In 2009 we genotyped 1,155 eggs from 35 untreated subjects from the pre-treatment collection and a total of 44 eggs from 6 treated subjects from the post-treatment collection, and finally a total of 4 eggs from 8 subjects from the 2-month post-treatment collection. In 2010 we genotyped 16 individual eggs and pools of eggs from 3 untreated subjects from the pre-treatment collection and 4 eggs from 2 treated subjects from the post-treatment collection. Treatment with ABZ resulted in a high ERR for hookworms at the group level: 98.6% (95% CI, 96.1-99.7) and 97.74% (95% CI, 38.43-100.00) in 2009 and 2010, respectively, and at the individual level: 90.08% and 97.01% in 2009 and 2010, respectively (Table 4.4).

We detected a SNP at codon position 167 in *A. lumbricoides* samples from the pre- and post-treatment collections from 2009 and 2010. We genotyped 471 individual eggs as well as pools of eggs from 14 untreated subjects taken from the pre-treatment collection in 2009 and found that the resistance-type (based on veterinary nematodes) allele "A" was present in 55.5% of cases. Two weeks after treatment, 164 eggs (individual and pools) were genotyped from 3 treated subjects and the frequency of allele A increased to 73.0% (p-value = 0.0005). Two months post-treatment, none of the subjects screened were positive for infection with

A. lumbricoides. In 2010 we genotyped 348 eggs (individuals and pools) from 10 untreated subjects from the pre-treatment collection, and the frequency of the resistance-type allele A was 49.7%. After treatment only two subjects were found to be positive for *A. lumbricoides*. The resistance-type allele was identified in 36.1% of the genotyped samples. The drug efficacy was high at both the group and individual levels in 2009 and 2010 (Table 4.4).

Table 4.4. Faecal egg count reduction rates in hookworms and *A. lumbricoides* detected by McMaster technique in 2009 and 2010. [1]ERR, egg reduction rate. The ERR estimates were obtained by dividing the difference between the arithmetic mean of the pre- and post-treatment FEC, by the mean of the pre-treatment mean count at [2]group level (ignoring individual variability) and at [3]individual subject level [4]CI, confidence interval. The confidence intervals of the egg reduction rate were calculated using a bootstrap resampling method.

	[1]ERR			
	[2]Group level (95% CI[4])		[3]Individual level (%)	
Parasites	2009	2010	2009	2010
Hookworm	98 (96.1-99.7)	97.7 (38.4-100.0)	90.1	97.0
A. lumbricoides	99.9 (99.5-100.0)	96.8 (65.1-100.0)	100.0	84.1

4.3.5. Drug efficacy and frequency of SNPs 198 and 200 in *T. trichiura* after one round of ABZ treatment

We investigated the association between the genotype frequency of β-tubulin gene SNPs at codon positions 198 and 200 and the response of *T. trichiura* to ABZ using the 2009 collection. In the good response group, drug efficacy calculated at the individual level was 100%, meaning that at the time of the post-treatment collection, infected subjects were all cured as no eggs were detected in

the feacal samples analyzed. From our data, 50% of the population analyzed showed a good response to ABZ treatment while the other half showed either an intermediate or poor response (Table 4.5).

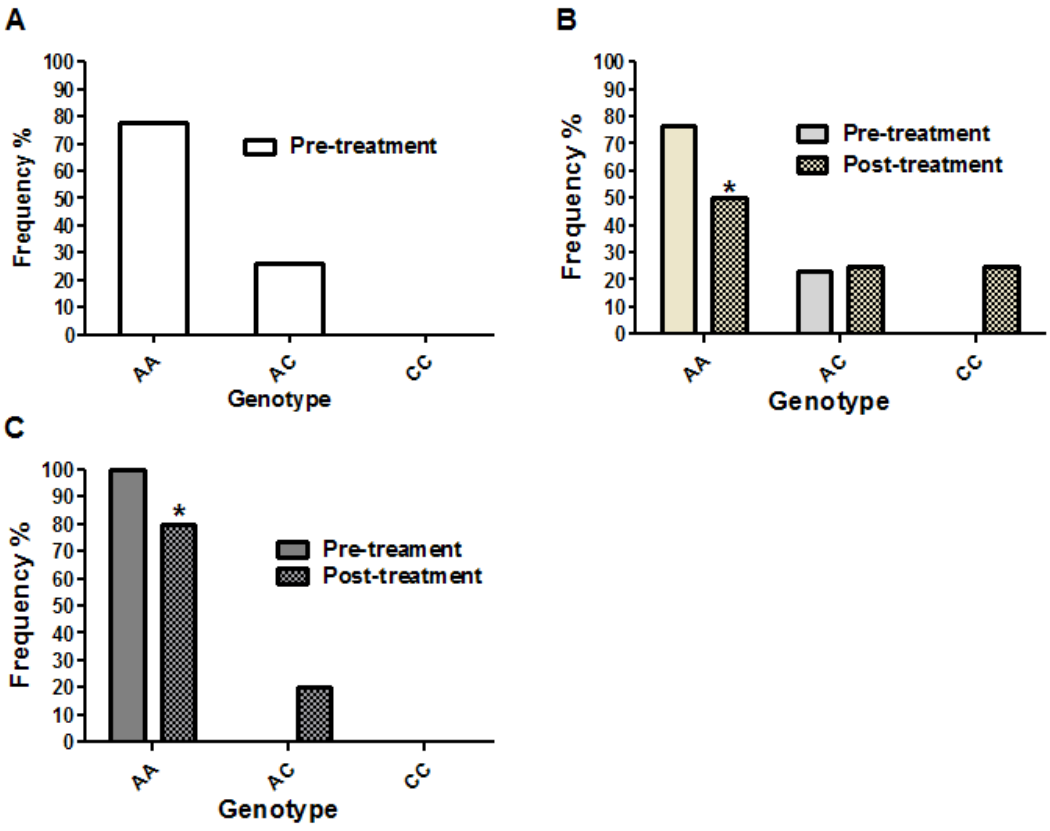
Table 4. 5. Response of *T. trichiura* to one round of ABZ treatment. [1]The ERR was calculated at individual level. [2]The FEC pre-treatment was greater than the FEC post-treatment. [3]Baseline epg, calculated before ABZ administration.

	Good response	**Intermediate response**	**Poor response**
% (n)	52.2 (18)	15.62 (5)	28.12 (9)
[1]ERR (%)	100	[60-80]	[2]0 or >0
[3]EPG mean ± SE	353.9 ± 153.6	530 ± 220.4	628.6 ± 259.1

One hundred and seventeen individual and pools of eggs were genotyped from 36 untreated subjects using samples from the 2009 pre-treatment collection. The susceptible genotype at codon positions 198 (GAA/GAA) and 200 (TTC/TTC) was predominant in the 3 response groups, followed by the heterozygous and finally by the homozygous resistance-type. Two weeks post-treatment, we genotyped 106 eggs (individual and pools) from 14 subjects. We identified the presence of a SNP at codon 198 as the homozygous resistance-type (25%) and as heterozygous (25%) in the intermediate response group, whereas pre-treatment only the SNP was present as heterozygote, and no significant increase was detected for the heterozygote. In the poor response group, the SNP was detected, as heterozygote (20%) while at pre treatment all the sample genotypes were

homozygote susceptible-type (Figure 4.4). The resistance-type allele C at codon 198 was identified in 62.5% of the intermediate response group and 34.1% of the poor response group (data not shown).

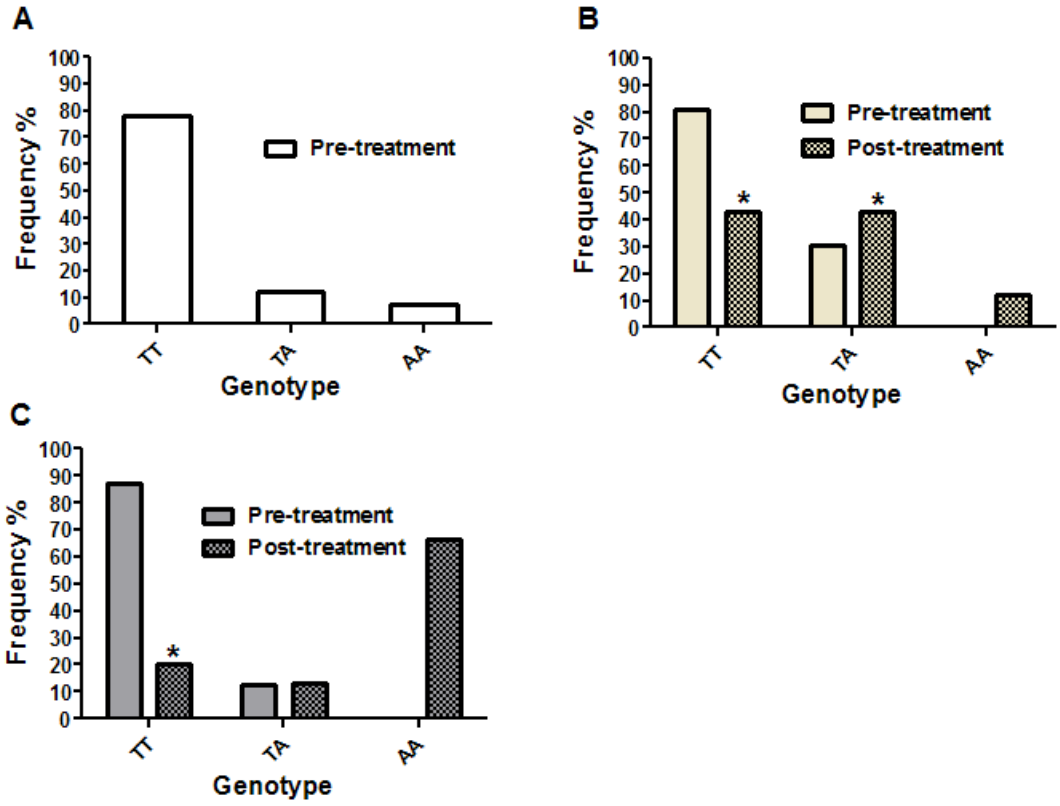
Figure 4. 4. Genotype frequency for the β-tubulin gene in *T. trichiura* at codon position 198 for the three response groups. Genotype frequency for the β-tubulin gene in the good (A), intermediate (B) and poor (C) response groups to ABZ. Sequences were diploid, AA indicates the homozygous susceptible-type GAA/GAA, AC the heterozygous GAA/GCA and CC, the homozygous resistance-type GCA/GCA. Tx=treatment. *Indicates a significant difference ($p<0.001$) in genotype frequency between the pre- and post treatment groups. P-values were obtained by Fisher's exact test.



A similar pattern was observed for codon 200 post-treatment in the intermediate and poor response groups. The SNP was present as the homozygous

resistance-type and heterozygous, in 43% and 70% of the population, respectively, while at pre-treatment, the resistance-type SNP was identified as being heterozygous in 30% of the samples analyzed. The increase in the resistance-type genotype at codon position 200 was significant ($p<0.001$). The resistance allele A was identified in 60.5% of the samples analyzed (data not shown). In the poor response group, we observed a shift in the distribution of the genotypes after treatment whereby the resistance-type (TAC/TAC) was the most abundant (70%) (Figure 4.5) and the resistance-allele A at codon 200 was present in 68.7% of the cases (data not shown).

Figure 4. 5. Genotype frequency for the β-tubulin gene in *T. trichiura* at codon position 200 for the three response groups. Genotype frequency for the β-tubulin gene in the good (A), intermediate (B) and poor (C) response groups to ABZ. Sequences were diploid. TT indicates the homozygous susceptible-type TTC/TTC, TA the heterozygous TTC/TAC and AA, the homozygous resistance-type TAC/TAC. Tx=treatment. *Indicates a significant difference ($p<0.001$) in genotype frequency between the pre- and post treatment groups. P-values were obtained by Fisher's exact test.



We genotyped 45 samples (individual eggs and pools) from 7 subjects collected two months post-treatment. We found that the same subjects infected with parasites and showing an intermediate or poor response to ABZ at the time of the 2009 collection were still infected with *T. trichiura* eggs. The genetic profiles of the eggs obtained from the intermediate responding individuals showed that 32.7% of eggs were carrying the resistance-type allele C at codon position

198 and 66.3% allele A at codon position 200. Similar findings were observed in the poor response group, with the resistance-type allele C at codon position 198 and the resistance-type allele A at codon position 200 occurring in 25.0% and 75.0% of eggs, respectively (data not shown).

We genotyped 16 samples (individual eggs and pools) from 3 subjects taken from the pre-treatment collection in 2010 (1 year post-treatment 1). The resistance allele C (198) was observed in 16.7%, and A (200) in 83.3 % (data not shown) of the eggs. The collected eggs were all obtained in 2009 from individuals belonging to the poor response group. The post-treatment genotype frequency for the β-tubulin gene is not presented because of the loss of follow-up of individual subjects classified in the different response groups.

4.4. Discussion

This study combined an epidemiological and molecular analysis of the three main STHs collected in the west and southeast regions of Haiti. The epidemiological study determined the dynamics of infection of *A. lumbricoides*, *T. trichiura* and hookworms and the molecular analysis revealed a possible explanation for the mediocre ABZ efficacy against *T. trichiura*.

The first objective of the study was to assess the prevalence of the main STHs in selected endemic communities. After two cycles of MDA, the prevalence and intensity of *A. lumbricoides, T. trichiura* and hookworms were reduced in the five communities. A similar reduction was observed in the 4 rural communities and in the unique urban one. It was noted that one year after treatment the prevalence of the three STHs was lower than previously described in other studies in Haiti [6] or in other countries [16], where one year post-treatment the prevalence returned to the previous baseline level. Several explanations could account for this decrease. First of all, the 2010 collection was performed 6 months after the earthquake and at that time the country and especially the areas where our communities were located were still in an extreme emergency situation. Mobile clinics and field hospitals were deployed in these areas so inhabitants of the communities had good access to health care even in the most remote communities. In addition, all medications, including antiparasitic ones, were free. We believe that the presence of a high density of post-earthquake health facilities positively influenced the decreased prevalence of STHs. Furthermore, in 2010 we added to the questionnaire a question on the use of traditional plants that cure

intestinal worm infections. The questionnaire revealed that many participants drank herbal infusions composed of medicinal leaves recommended by traditional physicians as preventative of worm infections or to treat stomach aches. Another study had scientifically demonstrated that some plants used in Haitian traditional medicine showed AH activity [17]. Thus, the use of these traditional plants could also have had an impact on reducing the prevalence of infection. Finally, after the pre-and post-treatment collections in 2009, participants were informed of the prevalence of STHs in their communities and at the same time we conducted a preventative hygiene program in which we also clearly explained to individual subjects the importance of ABZ compliance. This awareness of the benefits of the treatment may have influenced their behaviour.

Our second objective was to determine the risk of infections with *A. lumbricoides, T. trichiura* and hookworms. Our data revealed the importance of considering behavioural features and environmental sanitary measures of the studied population and sites. Indeed, only rural communities were included as there was more heterogeneity in the tested variables than within the urban community.

First of all, as already noted in other studies, there was a potential association between farming work and an increased risk of infection with STHs [18-20]. This may be related to the closer contact of farmers with the soil where the *A. lumbricoides* fertile embryonated eggs and hookworm filariform larvae, the infective stages, live and so could be accidently ingested by the human host or penetrate their skin [21]. Moreover, it is well known that *A. lumbricoides* eggs are

very resistant to extreme conditions and, therefore, can survive in the environment for many years [22]. Thus, this particular feature is an important factor that favours transmission. In addition, during the study it had been noticed that a large proportion of the participants, including the farmers, walked barefoot (data not shown), which could enhance the risk of transmission, as previously observed [23-25].

Secondly, we also confirmed that an increased density of people living in houses was positively associated with the risk of infection with *T. trichiura* [25-27]. Indeed it was common to find that people living within the same household were infected with the same parasite, and our model considered the household cluster. The crowding in the household and non-access to a latrine are factors that may favour transmission within the house. Although, the lack of access to latrines was not identified as a risk factor for infection in our study, it may still indirectly contribute to the risk of acquiring STHs. Finally, gender was a significant risk factor for infection with *T. trichiura*. The reason for this could be that males were more often assigned to agricultural activities, necessitating close contact with the soil, whereas women were more likely to be street vendors.

Thus, our findings suggest that there is an urgent need in the studied communities to set up a rural planning policy in order to interrupt the viscous cycle of transmission. The lack of latrine access leads to defecation in the surrounding environment that could contaminate the soil with helminth eggs. Farmers, mainly males, are more exposed to the risk of encountering STHs by the

nature of their work that requires close contact with soil and also by their behaviour (walking barefoot).

Our final objective was to identify SNPs in STH β-tubulin genes and determine the association with ABZ efficacy. In a previous study (Chapter 3) we had already found the presence of a SNP at codon 167 in individual *A. lumbricoides* eggs collected before and after ABZ treatment in 2009. The genomic analysis of eggs collected in 2009 and 2010 was consistent with the earlier data in that we did not find an association between the presence of SNP at codon position 167 and ABZ efficacy. Indeed, the ERR at the individual and group levels were well within the range normally observed with the drug [28].

In contrast, in *T. trichiura* we found phenotypic and genotypic evidence of an association between the presence of SNPs at codon positions 198 and 200 and the poor efficacy of ABZ. Our results showed that there was variation in the response to ABZ among the treated participants in 2009.

The good, intermediate and poor response groups refers to the response of individuals harbouring *T. trichiura* eggs after one round of ABZ treatment. In the good response group, 2 weeks after treatment individuals were completely cleared of eggs, whereas in the poor response group no reduction was identified or surprisingly the FEC post-treatment was higher than the FEC pre-treatment. Subjects in the poor response group harboured higher epg, measured in pre-treatment collection samples, than individuals in the good response group. Thus, this data may suggest that for *T. trichiura* there is an association between pre-

treatment FEC and treatment efficacy, a finding that is consistent with a previous study [29].

Genotypic analysis of eggs from pre-treatment collection samples revealed that in the good and low response groups, the susceptible-type genotypes at codon positions 198 and 200 were predominant. The resistance-type genotypes (heterozygous and homozygous resistance types) were not exclusively associated with eggs obtained from individual subjects showing poor ABZ responses. Thus, for pre-treatment collection samples, the resistance-type genotypes seemed to be homogenously distributed within the good and poor response groups and so seemed not to be an essential prerequisite that determines the poor response to ABZ. In addition, in the intermediate response group the resistance allele A at codon position 200 was identified in 19.2 % of the pre-treatment samples; that was the highest frequency among the three response groups. This confirmed the non-association between the resistance-allele or resistance-type genotype and the resistance phenotype in pre-treatment samples. It is not surprising as it has been demonstrated that resistance-type alleles or alleles conferring resistance are likely to be present in non-treated populations [10,30,31].

However, after treatment, we identified a significant increase in resistance-type genotypes at codon positions 198 and 200 in the intermediate and poor response groups, which was much more pronounced (see results) for codon position 200 than for 198. This result is consistent with our previous finding (Chapter 3), showing that the SNP at codon position 198 seems to be involved in the selection process, but has less impact than the SNP at codon position 200 in

conferring resistance. In veterinary parasites, it has been observed that a SNP at position 198 is rarely encountered [32].

We also examined the SNPs at 198 and 200 in samples collected at 2 months and at 1 year post-treatment. Surprisingly, 2 months after treatment, *T. trichiura* eggs carrying the resistance-type allele C (198) or A (200) were all from individual subjects previously classified in the intermediate and poor response groups. This result strongly suggests that it is more likely a persistence of the parasite carrying the resistance-type allele than a rapid reinfection. Consistently, one year post-treatment, the resistance alleles were found in the population but only in individual subjects who responded poorly to ABZ during the previous treatment. These results suggest that the resistance allele remains in the populations at a moderate frequency.

It is important to recognize that the study had some limitations. First, the loss of follow-up for some individuals at the post-treatment collections considerably reduced the sample size when assessing the ERR for individual and also led to the exclusion of all participants from the urban community from the logistic models. Indeed, as previously explained, the earthquake caused the relocation of participants from the urban community, so it prevented 1-year post-treatment follow-up. However, this loss of participants did not impact our results too much as the baseline prevalence and intensity of STHs in the urban community was low and the results were consistent in 2010 even with new participants. In addition, for some collections, the sample size of the genotyped

eggs was low. This was due to experimental failure, especially with individual eggs.

However, despite the limitations mentioned above, this study highlights the benefit of combining epidemiological and molecular analysis when monitoring STH infections. Indeed, analyzing the risk factors of infection with STHs allowed for the evaluation of how the individual, environment and household factors may influence the dynamics of infection and reinfection with STHs inside a community [33,34]. Thus, these characteristics can be taken into account to identify potential additional measures other than drug treatment that could be applied. Indeed, the reduced use of the same AH may be an effective strategy to delay the development of resistance [35] that is a growing concern for the control of STHs. Various studies have reported a sub-optimal response in STHs, especially in *T. trichiura* [36,37] and in this current work we showed evidence of the association between genetic changes due to SNPs at codons 200 and 198 in the β-tubulin gene and intermediate and low ABZ responses. Thus, this link between genotypic and phenotypic resistance makes the SNPs at positions 198 and 200 good candidate markers for resistance surveillance.

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CHAPTER FIVE

General discussion

The WHO has recognized STH infections as being a major neglected tropical disease (NTD). The WHO launched a new roadmap for reducing or eliminating the number of NTDs by 2020. In the London Declaration on NTDs, global health agencies, pharmaceutical companies and politicians from various countries have committed to support the implementation of this guideline for combating NTDs [1]. In order to reach the goal of reducing the morbidity from NTDs by 2020, it has been proposed to periodically distribute through MDA one of two BZ drugs, ABZ or MBZ [2]. Thus, control programs for STHs rely mainly on repeated drug treatments that make the programs highly vulnerable should BZ resistance develop and spread [3]. From experience with veterinary parasites, it is well known that repeated treatment with the same class of AH drug can lead to the development of resistance, caused by SNPs in the β-tubulin gene [4]. Furthermore, in human filarial nematodes some studies have also reported the emergence of drug resistance or drug resistance polymorphisms after different treatment regiments with ABZ or ivermectin (IVM) [5-7]. To monitor drug efficacy in STHs, the FEC reduction test (FECRT), the cure rate (CR) and the biological test known as the EHA are used and provide an indication of AH drug resistance [8]. These tests are simple, cost-effective and suitable to use in field situations. However, they lack sensitivity and are not standardized for STHs. Therefore, there is an urgent need to develop sensitive diagnostic tools for STHs in order to detect at an early stage genetic changes associated with ABZ/MBZ resistance and prevent the dissemination of the resistance phenotype.

The main purpose of this thesis was to provide an overview of the current situation with regard to the development of resistance in STHs by collecting samples from three different countries located in the Caribbean, Africa and Central America. To our knowledge, this is the only multi-site study that has performed genetic analysis on eggs collected prior to and after one round of ABZ treatment in the same populations. For the first time, we also examined the effect of two rounds of ABZ on polymorphisms in Haitian endemic communities naive for MDA with ABZ treatment.

In the second chapter of the thesis, we identified new elements to improve the biological EHA for human hookworms. Indeed, we demonstrated that the EHA could first be optimized on the dog hookworm, *Ancylostoma caninum,* which shares characteristics with the related human hookworms *Ancylostoma duodenale* and *Necator americanus* [9]. This canine hookworm is highly prevalent in the United States especially in regions where soil and climate conditions are optimal for the development and persistence of larvae [10], while human hookworms are only found in tropical countries. Consequently, experiments involving the EHA for human hookworms necessarily require travel to and in the affected regions. The easy access and rapid culture of *A. caninum* eggs allowed us to perform a variety of *in vitro* qualitative experiments to determine the optimal conditions for assessing the drug response.

Based on the experiments on *A. caninum* we proposed an easy and cost effective way to preserve stool samples anaerobically. This aspect is important to consider when performing large-scale stool sample collection in the field since

eggs hatch rapidly into larvae if not processed within hours following collection. It is, therefore, necessary to preserve eggs at this stage in order to perform the EHA. Anaerobic preservation in an airtight container can prevent the hatching of eggs for one week and, based on our experiments, it did not negatively impact the dose response. In addition, we identified solutions that significantly improved the dose response. Application of the antifungal Amphotericin B solution before the beginning of the assay had a direct impact on the dose response by reducing the opacity caused by fungal growth, while addition of staining and destaining solutions at the end of the assay indirectly improved the dose response by facilitating the scoring of eggs and larvae. All these conditions were reproducible on human hookworms in a field lab with few resources, confirming that *A. caninum* is a good model for developing and optimizing assays that can then be transferred to human hookworms [9].

In this chapter we also developed sensitive molecular markers for β-tubulin gene codons 167, 198 and 200 in *A. lumbricoides, T. trichiura* and hookworms, for monitoring resistance selection in these harmful parasites. We identified a polymorphism at codon position 200 in pools of *N. americanus* eggs collected in Haiti, in which 36% of the genotyped eggs carried the resistance-type allele A. We did not have information on the treatment history of the individual subjects involved in the study, however, we know that the samples were collected in an area where the national program to eliminate LF has been implemented for many years with the population receiving DEC in combination with ABZ annually [11]. It would have been interesting to assess and compare ABZ efficacy prior to the

implementation of the control program and after several rounds of treatment to see how ABZ may have influenced the frequency of the polymorphism. Interestingly, it has been found that the frequency of the polymorphism at codon 200 in the filarial parasite *Wuchereria bancrofti* significantly increased after one or two rounds of ABZ treatment [12]. In the context of regular MDA with the same drug, we believe that the same pattern could occur in hookworms. Thus, it would be useful to design mathematical models to predict the spread of resistance according to the frequency of the resistance-associated SNP. This important tool is available for some filarial parasites [5], and it is important to consider the resistance allele frequency when implementing a control strategy. For STHs, a mathematical model is not yet available, consequently the regular assessment of SNP frequencies prior to and after ABZ treatment is necessary in endemic regions under treatment. This particular aspect was examined in the third chapter of the thesis.

In this chapter, our main finding was the clear evidence of selection of resistance in the whipworm *Trichuris trichiura.* Indeed, a significant increase of the homozygote resistance-type genotype was identified in individual eggs after ABZ treatment. This finding was consistent in samples collected from two different sites (Kenya and Haiti) and populations (only children in Kenya while adults and children in Haiti) with different treatment histories (previously treated in Kenya and naive in Haiti), suggesting that resistance is not selectively developing in only one area or affecting a particular population. However, the common feature of the sites in Kenya and Haiti was that the samples were

collected for LF control programs and, therefore, the parasitic populations could have previously been under selection by exposure to ABZ. In addition to confirming the presence of the polymorphism at codon 200 in the β-tubulin gene increased with treatment, we tested whether the genotype frequency was in Hardy Weinberg equilibrium (HWE) before and after treatment. This test revealed that in Haiti, the increase of the homozygous resistance-type after treatment correlated with a departure from HWE as illustrated by a deficiency of heterozygous and an excess of homozygous alleles.

It is important to highlight that resistance to BZ drugs is usually recessive [13], meaning that an excess of homozygosity will be expected to accompany the development of resistance. In Kenya, this polymorphism in codon 200 was not in HWE before treatment, characterized by an excess of homozygotes. Different hypotheses may explain the HWE imbalance in genotype frequencies. As described in other studies, the presence of a null allele could be responsible for the deficiency of heterozygotes [14]. However, null allele frequency estimates demonstrated that there was no significant evidence of its presence. Hypotheses, other than resistance selection that could cause a deficiency of heterozygotes include non-random mating caused by inbreeding and genetic drift resulting in bottlenecking. Both hypotheses are unlikely because of the characteristics of the life cycle of *T. trichiura* where eggs *in refugia* (parasites in the soil not exposed to treatment) should result in high genetic flow in the population. It was, therefore, concluded that the paucity of heterozygotes was likely caused by a pre-existing

selection in Kenya, possibly due to an ongoing MDA for STHs [15] and LF control in the regions examined.

We also identified a significant increase of the homozygote resistance-type genotype at codon position 198 after treatment in Haiti, suggesting that this SNP could also be involved in resistance selection. In Haiti and Kenya the efficacy of ABZ was moderate and, therefore, suggested that the SNP found may be responsible for this less than optimal response. At both sites, drug quality was assured and so could not be considered as a confounding variable. In this chapter we also identified a polymorphism at codon 200 in hookworms from Kenya at a low frequency pre- and post-treatment while drug efficacy was high. This polymorphism seemed to have not yet affected the drug response. However, this finding is a warning for the possible development of phenotypic resistance in hookworms. In *A. lumbricoides,* we found a polymorphism at codon 167 in samples collected from the three sites before and after treatment, nevertheless the treatment did not change the SNP frequency. Thus, the SNP at codon 167 does not appear to be a marker for resistance in this parasite.

In the fourth chapter, the longitudinal epidemiological study not only confirmed the genetic selection in *T. trichiura* but it also correlated with the phenotypic response. By classifying individual subjects into ABZ drug response groups, two important characteristics were identified. First, the pre-treatment egg count of *T. trichiura* varied in the three response groups (good, intermediate and poor). The poor response group was associated with the highest *T. trichiura* intensity whereas in the good response group the lowest parasite intensity was

detected which may imply that the response to ABZ depends in part on the pre-treatment egg count. This hypothesis has previously been highlighted in another study where they showed that the intensity of the pre-treatment egg count influences the ERR when assessing drug efficacy [16]. This information suggests that for high intensity infections, multiple BZ doses will increase efficacy. However, it has been demonstrated in the veterinary nematode *Haemonchus contortus* that the increase of ABZ dose rate confers a higher selection pressure in the β-tubulin gene [17]. Secondly, we found a significant increase in the resistance alleles "A" and "C" for codons 200 and 198, respectively, in the intermediate and poor response groups after one round of treatment. Thus, based on this result, we do not recommend increasing the ABZ dose rate to treat *T. trichiura*. Also, parasites carrying both resistance alleles "A" and "C" survived the treatment and persisted for two months after treatment in the intermediate and poor response groups. It was interesting to note that both alleles were still present at moderate frequency in the poor response group one year after treatment.

This study reports for the first time the genotypic and phenotypic selection in *T. trichiura.* Since few effective drugs are available against *T. trichiura* and BZ selection seems to already be established, in order to sustainably decrease the prevalence and morbidity of this parasite we propose to implement an intervention based on prevention of transmission (prevention by wearing shoes, latrine access, and hygiene) rather than relying solely on a drug-based strategy. In this study, we also identified the main risk factors for infection with STHs. This analysis

combined with the molecular data provides key elements for monitoring resistance and control of STHs in an endemic country.

However, it is important to recognize that the study has several limitations. The lack of knowledge of field resistant hookworm isolates prevented us from validating the EHA for human hookworms as a diagnostic tool for BZ resistance. Also, the low infection intensity in the field was a weakness of the study, since few assays were performed and, therefore, a general conclusion regarding the drug response could not be made. In addition, the pyrosequencing demonstrated its ability and sensitivity to detect SNPs in STHs; however, the technique requires sophisticated equipment and expensive reagents, therefore limiting its application in low resource countries where STHs are endemic. An alternative approach could be the application of Smart Amplification. Indeed, this isothermal DNA amplification method allows for the rapid detection of SNPs without performing DNA extraction or PCR amplification [18]. The SmartAmp2 is a polymerase-based chain reaction that requires a unique primer design and a strand-displacing polymerase that enables the amplification of the target DNA sequence under isothermal conditions. As background suppression is achieved by the mismatch-binding protein Taq MutS, the target SNP is precisely detected by isothermal DNA amplification [19]. This method has been applied to human clinical studies and revealed the ability to rapidly distinguish the mutant genotype from that of the wild type with high sensitivity [20]. This may enable the selection of the most appropriate therapeutic strategy. For human STHs, the SmartAmp2 assay is under

development [21], thus this technique should be suitable for the field as it could be performed in one reaction and be cost effective.

Another limitation of the study was the low sample size of the genotyped eggs due to technical failure including failure of the DNA extraction, PCR amplification and pyrosequencing of some samples. Eggs had been preserved in alcohol for several months before the DNA was extracted. The egg suspension was contaminated with faecal debris and the large number of samples prevented us from performing a clean isolation of the recovered eggs. Thus, it may be possible that some substances from the feacal material inhibited the DNA extraction and amplification. DNA extraction of fresh material may have improved these steps. Also, the failures were more pronounced for single eggs where the DNA concentration was very low (<5ng/μl). This may have restricted PCR amplification even after two PCR rounds and affected the pyrosequencing.

The highest rate of PCR failure was recorded for *T. trichiura*. In this parasite, visual inspection under the microscope revealed that the addition of lysis buffer was not sufficient to disrupt the eggshell. Freezing at -80°C prior to heating at 60°C helped considerably in disrupting the egg's integrity. However, additional procedures (e.g. applying pressure on eggs) were required to break them on some occasions, with eggs still failing to rupture on other occasions. These additional procedures were time consuming and did not always succeed. Furthermore, the design of some primers in intronic regions may have contributed to an increase in the failure rate not only for *T. trichiura* but also for *A. lumbricoides* and hookworms where experimental failure was also recorded. Polymorphisms in

introns where amplification primers were meant to bind may have decreased amplification rates. This may have biased the frequencies of the SNPs of interest since it is has been shown (in humans) that polymorphisms in an intron may be associated with polymorphisms in an adjacent exon [22].

Furthermore, another possible weakness in the study was the non-homogeneity of the multi-site study design. As previously discussed in the thesis, we did not establish a standard protocol for the populations selected with respect to stool collection and procedures for determining egg counts at all sites; we were dependent on collaborators involved in national MDA programs. Thus, the different protocols applied at each site may have influenced the results with regard to the observed infection and intensity, depending on the population sampled (children or adults) [23], and drug efficacy, depending on the sensitivity of the diagnostic technique used to count the eggs (FLOTAC, McMaster or Kato-Katz) [24-26]. In spite of these differences our main goal of assessing SNP frequency before and after ABZ treatment was achieved and the resulting data was consistent and comparable between the three sites.

Finally, the earthquake in Haiti in 2010 influenced the population sample size of the longitudinal study. It specifically reduced the number of participants in the urban community that were the most affected by the earthquake. Also, the loss of equipment (microscopes) and vehicles considerably slowed the field work and also reduced the number of participants since the sampling days or times could not be defined according to the optimal time to reach the maximum number of people but were rather according to the number of available vehicles. In addition,

the rainy season disrupted the collection of samples in the most remote communities.

Taken together, our results provide important data for the control of human STHs. Indeed, in some consortiums on STHs, it has been proposed to include in their control programs the elaboration of appropriate diagnostic tests for detecting and monitoring drug resistance [27,28]. In this thesis, we presented molecular diagnostic tools for routine monitoring of resistance in the three most common human STH species. The protocols for the various tests (DNA extraction, primer design, pyrosequencing, and data analysis) that were also adapted to the particular parasite stage were described in detail to allow their use in other studies. Indeed, we optimized the tests for adult worms, larvae, single eggs or pools of eggs by taking into account the different experimental variables (DNA concentration, preservation of material, etc). Furthermore, our data provided an important warning of the possible development of resistance in hookworms and evidence of resistance in *T. trichiura*. These findings highlight the necessity of establishing a close collaboration between scientific researchers and control program managers that could improve control strategies. Furthermore, the epidemiological study presented baseline information that also needs to be considered when planning to implement a control program or could even help in modifying the control strategy accordingly. Currently there is no "gold standard" control strategy for STHs that can effectively reduce the prevalence and morbidity and prevent reinfection at the same time delay the possible development of drug resistance. In this thesis we provided relevant elements for addressing this problem.

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INTRODUCTION TO APPENDICES

Two appendices are included in the thesis:

Appendix A presents a manuscript on the development of diagnostic tools to detect single nucleotide polymorphisms in *Trichuris trichiura* and *Ascaris lumbricoides* that could be associated with benzimidazole resistance.

Appendix B shows the supplemental table for the second manuscript (Chapter three).

APPENDIX A

Development of diagnostic tools to detect a single nucleotide polymorphism, in *Trichuris trichiura* and *Ascaris lumbricoides*, that could be associated with benzimidazole resistance

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Abstract

Background: The soil-transmitted helminths (STH), Ascaris lumbricoides and Trichuris trichiura, are gastrointestinal parasites causing many disabilities to humans, particularly children. The benzimidazole (BZ) drugs, albendazole (ALB) and mebendazole (MBZ) are commonly used for mass treatment for STH. Unfortunately, there is concern that increased use of anthelmintics could select for resistant populations of these human parasites. In veterinary parasites, and lately in filarial nematodes, a single amino acid substitution from phenylalanine to tyrosine, known to be associated with benzimidazole resistance, has been found in parasite β-tubulin at position 200. We have developed pyrosequencer assays for codon 200 (TTC or TAC) in Ascaris lumbricoides and Trichuris trichiura to screen for this single nucleotide polymorphism (SNP).

Method and findings: Pyrosequencing assays were developed and evaluated for detecting the TTC or TAC SNP at codon 200 in β-tubulin in A. lumbricoides and T. trichiura. Genomic DNA from individual worms, eggs isolated from individual adult worms or from feacal samples with known treatment history and origin were sequenced at β-tubulin by pyrosequencing and genotypes confirmed by conventional sequencing. The assays were applied to adult worms from a benzimidazole-naïve population in Kenya. Following this, these assays were applied to individual worms and pooled eggs from people in East Africa (Uganda and Zanzibar) and Central America (Panama) where mass anthelmintic drug programs had been implemented. All A. lumbricoides samples were TTC. However, we found 0.4% homozygous TAC/TAC in *T. trichiura* worms from

non-treated people in Kenya, and 63% of T. trichiura egg pools from treated people in Panama contained only TAC.

Conclusion: While the codon 200 TAC SNP was not found in any of the *A. lumbricoides* samples analyzed, a rapid genotyping assay has been developed which can be used to examine larger populations of this parasite and to monitor for possible benzimidazole resistance development. The TAC SNP at codon 200, associated with benzimidazole resistance in other nematodes, does occur in *T. trichiura* and a rapid assay has been developed to allow populations of this parasite to be monitored for the frequency of this SNP. Sample sizes were small, anthelmintic efficacy was not assessed, and treated and non-treated samples were from different locations, so that these frequencies cannot be extrapolated to other populations of *T. trichiura* or to a conclusion about resistance to treatment. The occurrence of the TAC SNP at codon 200 of β-tubulin in *T. trichiura* may explain why benzimidazole anthelmintics are not always highly effective against this species of STH. These assays will be useful in assessing appropriate treatment in areas of high *T. trichiura* prevalence and in monitoring for possible resistance development in these STHs.

Author summary

Soil-transmitted helminths, *Ascaris lumbricoides* and *Trichuris trichiura,* are gastrointestinal nematodes causing many disabilities in tropical parts of the developing world. Control programs such as "The Focussing Resources on Effective School Health" (FRESH) Partnership have been implemented to remove human soil transmitted nematodes through large-scale use of benzimidazole anthelmintic drugs for school-aged children in developing countries. The benzimidazole drugs, albendazole and mebendazole, are commonly used as a single annual treatment in areas where the burden is high. In veterinary nematodes, repeated use of these anthelmintics has selected for resistant populations. Resistance to benzimidazoles is commonly associated with a single amino acid substitution from phenylalanine to tyrosine in β-tubulin gene at position 200. In this study, we have developed pyrosequencing assays for the codon 200 in *Ascaris lumbricoides* and *Trichuris trichiura* to screen for this single nucleotide polymorphism (SNP) in β-tubulin. The 200Tyr SNP was detected at low frequency in *T. trichiura* from non-treated people from Kenya and at high frequency in *T. trichiura* from treated people from Panama. The presence of the resistance-associated SNP may play a role in the sometimes low and variable efficacy of benzimidazole anthelmintics against *T. trichiura*.

A.1. Introduction

The soil-transmitted helminths (STH) are gastrointestinal nematodes widely distributed throughout the tropical and subtropical parts of the developing world. More than a billion people are infected with at least one species and 300 million are estimated to have severe infections with more than one of these parasites [1]. Infection often causes chronic disability but in certain instances may precipitate death. School age children are the most at risk of infection with STH and can show symptoms of malnourishment, experience growth stunting and intellectual retardation, with cognitive and educational deficits [1]. Control programs such as the ”Focussing Resources on Effective School Health” partnership (FRESH) have been implemented in endemic countries to reduce the morbidity of school-aged children by using a single annual treatment with benzimidazole drugs : either albendazole or mebendazole [2].

BZ drugs are broad spectrum anthelmintics that bind to β-tubulin, causing interference with tubulin polymerization and destabilization of microtubules [3]. Mass drug administration (MDA) programs reduce the incidence and intensity of infections; however they can also cause a selection pressure on the parasites to develop resistance. Many studies have demonstrated that the widespread and frequent use of anthelmintic drugs in veterinary nematodes has led to the development of resistance [4,5]. This resistance is usually due to a single nucleotide polymorphism (SNP) which causes an amino acid substitution from phenylalanine (Phe, TTC) to tyrosine (Tyr, TAC) in parasite β-tubulin at codon 200 [6]. A similar SNP at codon 167 (Phe167Tyr) or a glutamate to alanine

change at codon 198 (Glu198Ala) can also occasionally be associated with benzimidazole resistance [7]. In human STH, and especially in hookworms, reports have suggested the development of benzimidazole resistance but failed to provide conclusive evidence [8]. If resistance against albendazole and mebendazole occurs, it will be a major threat to the mass de-worming programs in developing countries.

The objectives of this research were: (i) to determine the genomic sequences of the β-tubulin around codon 200 in *A. lumbricoides* and *T. trichiura*, (ii) to develop pyrosequencing assays for the detection of phenylalanine 200 or tyrosine 200 in the β-tubulin gene of each of these nematodes, and (iii) to assay DNA of individual adult worms and pooled eggs from the field to determine whether the Phe200Tyr SNP can be found and to obtain initial assessments of the frequency in areas that are either naïve to benzimidazole treatment or have been subject to benzimidazole treatment.

A.2. Materials and methods

A.2.1. Parasite material

In this study, all parasite samples were from school-age children who have been infected naturally by *A. lumbricoides* and/or *T. trichiura*. Samples of adult worms and/or eggs were collected from different locations with different treatment histories. Individual adult worms, 39 *T. trichiura* (20 males and 19 females) and 38 *A. lumbricoides* (19 males and 19 females), were collected from

children from Kisumu, Kenya (latitude: 00°03' S, longitude: 35°5' E). These children were naïve for anthelmintic treatment; however, they received a single dose of "Combantrin®" (pyrantel) in order to expel the adult worms in feaces. These worms were considered as control parasites and stored at -80°C until needed.

Faecal samples with *A. lumbricoides* and *T. trichiura* eggs were collected in Panama in the Comarca Ngobe Bugle region (latitude: 8°3' N, longitude: 86°12' W) from 29 children in 3 different schools. These children had received a single dose of ALB (400 mg). Prior treatment, before commencement of the study, may have occurred, but was not documented. Stool samples were preserved in 70% alcohol and stored at 4°C.

From another study carried out in Zanzibar and Uganda [9], we received 91 DNA samples from individual *A. lumbricoides* adult worms recovered after being expelled with "Combantrin®" from patients who lived within areas where large scale interventions with benzimidazoles were ongoing. The DNA was preserved in alcohol and stored at 4°C.

A.2.2. *A. lumbricoides* and *T. trichiura* egg recovery and DNA extraction

The DNA assay was applied to eggs from non-treated (BZ-naïve subjects) and eggs from subjects in treated areas. *A. lumbricoides* eggs from Kenya were recovered from the uterus of adult female worms. Before the dissection of each female worm, the anterior end of the parasite was separated from the body to

avoid contamination of adult DNA with DNA from eggs. Female worms were then opened longitudinally. At approximately one quarter length along the body, the uterus is attached to the genital pore and subsequently divides into two branches. Each uterine branch was cut and opened to release the eggs. From the study carried out in Panama, 29 pooled *A. lumbricoides* egg samples and 8 pooled *T. trichiura* egg samples were recovered from stool samples by using a flotation technique [10].

Following the recovery of eggs, DNA was extracted from pooled *A. lumbricoides* eggs collected from the uterus of adult female worms. It was also extracted from individual *A. lumbricoides* female and male worms and from individual *T. trichiura* adult worms using the DNeasy® Blood and Tissue Extraction Kit (Qiagen) according to the manufacturer's protocol. For the DNA extraction of *A. lumbricoides* and *T. trichiura* eggs from stools samples from Panama, the QIAamp® DNA stool mini kit (Qiagen) was used according to the manufacturer's protocol.

A.2.3. Determination of the β-tubulin sequence in *A. lumbricoides* adult worms

Genomic sequence (GenBank AF034219) was available for *T. trichiura* β-tubulin, [11], but was not available for *A. lumbricoides*, so it was necessary to generate this sequence. Total RNA was extracted from adult *A. lumbricoides* from Kenya by TRIzol Reagent (Invitrogen™, Life Technologies, Burlington, ON) according to the manufacturer's protocol. Then, the total RNA was reverse

transcribed with the oligo-dt (12-18) primer according to the manufacturer's instruction.

For the initial isolation of the *A. lumbricoides* β-tubulin gene, cDNA was amplified with degenerate primers. These primers were designed based on the conserved region of β-tubulin of six related nematodes: *Haemonchus contortus* (GenBank, M76493 - isotype 1)*, Brugia malayi* (GenBank, AY705382), *Necator americanus* (GenBank, EF392851)*, Trichuris trichiura* (GenBank, AF118385), *Teladorsagia circumcincta* (GenBank, Z69258 - isotype 1) and *Onchocerca volvulus* (GenBank, AF01886). Two sets of primers were designed for a nested PCR approach. In the first round PCR, the cDNA was amplified with the outer sense primer (5'-3') CAAAGTGGAGCKGGHCACAACTGGC and the outer antisense primer (5'-3') CGBAGATCHGCATTCAGCTGHCCAGG. The PCR product from the first round was then used as a template for the subsequent amplification using the nested primers, sense (5'-3') CTYGGTGGAGGYACMGGWTC and antisense (5'-3') CGBAGATCHGCATTCAGCTGHCCAGG. For both rounds, the PCR conditions were an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 1 min and a final extension at 72°C for 5 min. Prior to cloning, a 5 µl aliquot of PCR product from the nested reaction was examined on a 1% agarose (TAE) electrophoresis gel to confirm the size of the product. The amplified PCR products were cloned into the pCR2.1 TOPO vector using a TOPO-TA-Cloning kit (Invitrogen™, Life Technologies, Burlington, ON), as per the manufacturer's instructions and then sequenced.

Based on the sequenced fragment, gene-specific primers were designed for the 5' and 3' rapid amplification cDNA ends (RACE) reactions. For the 5' RACE reaction two primers were designed, (5'-3') CGTTGAGCGCCCTGTATGC and (5'-3') CAACACCACATCAGAAACCT and used in a semi-nested PCR reaction with the nematode splice leader sequence SL1 (5'-3') GGTTTAATTACCCAAGTTTGAG [12]. The amplification conditions for the primary reaction were 3 min at 94°C, followed by 40 cycles at 94°C for 45 s, 59.4°C for 45 s, 68°C for 1 min and a final extension at 68°C for 5 min. For the semi-nested reaction the amplification conditions were as outlined above with an annealing temperature of 55.8°C.

To isolate the 3' end of β-tubulin cDNA, two gene specifics primers were designed (5'-3') CCACGTCTTCACTTCTTCATG and (5'-3') GTACGACATCTGCTTCAGGACCCTG, and used in a nested PCR reaction with oligo adaptor primers B_1 (3231)(5'-3') CCTCTGAAGGTTCACGGAT and B_2 (3232)(5'-3') CACGGATCCACATCTAGAT, respectively. The PCR conditions for both reactions were as described above with an annealing temperature of 60°C for the first reaction and 55.6°C for the nested reaction. The resulting fragments after the second round of each reaction were purified using the QIAGEN PCR purification kit® according to the manufacturer's protocol. They were subsequently ligated into pGEM-T cloning vector (Promega, Madison, WI) and sequenced from both directions with SP6 and T7 vector primers. Phylogenetic analysis was undertaken with the UPGMA method and performed

using the Mac vector program in order to identify the relationship between β-tubulin sequences of 13 nematodes.

A.2.4. Development of diagnostic tests in *A. lumbricoides* and *T. trichiura* benzimidazole naïve worms

To optimize the pyrosequencing DNA assay, two control plasmids were constructed for each species and contained either the "sensitive" codon TTC for the wild type or a mutation, generated by site-directed mutagenesis (TAC - mutant type), inserted at position 200. These plasmids were based on the amplification of a small portion of β-tubulin genomic sequence from *A. lumbricoides* and *T. trichiura* worms that were naïve for benzimidazole treatment. Gene-specific primers were designed to amplify a small fragment of genomic DNA (GenBank, FJ501301) surrounding the codon 200. In *A. lumbricoides*, primers were based on the sequence of the fragment of *A. lumbricoides* β-tubulin cDNA. All specific primers were designed with the software gene runner® in the exonic region, sense (5'-3') GGTGGAGGCACAGGATCTGGC, antisense (5'-3') GCAGCCGCTCCTCG. For *T. trichiura*, primers were directly designed from the genomic sequence (GenBank AF034219), sense (5'-3') GGTTTCAGATACAGTTGTAG (position 1212-1231, located 76 amino acids upstream of the first T of the codon 200 TTC) and antisense (5'-3') CAAATGATTTAAGTCTCCG (position 1356-1374, located 146 amino acids upstream of the first T of the codon 200 TTC). PCR reaction conditions were an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, an annealing temperature of 52°C in *A. lumbricoides* and 56°C in *T. trichiura*, for

45 s, and 68°C for 1 min and a final extension at 68°C for 5 min. Resulting fragments were cloned and sequenced as described above.

The site directed mutagenesis strategy consisted of the amplification of two overlapping fragments using outer and inner mutagenesis primer pairs. Then, the opposing PCR strands were annealed at overlapping regions, extended, and amplified by PCR to produce the desired full-length strand. Two pairs of primers were designed, in *A. lumbricoides* outer sense primers (5'-3') GTTTCTGATGTGGTGTTGGAG, antisense (5'-3') CAAATGGTTGAGGTCTCCG and inner mutagenesis primer, sense (5'-3') CGATGAAACCTACTGCATTGACAATG, antisense (5'-3') CAAATGGTTGAGGTCTCCG. The PCR conditions were as outlined above with an annealing temperature of 54°C. PCR products were electrophoresed though agarose gels and then purified. Ten µl (10 – 100 ng DNA) of each purified PCR product were mixed and denatured at 94°C for 3 min. The second PCR reaction had an annealing temperature of 53°C and allowed the production of the desired full-length strand. In *T. trichiura* the protocol used to generate the mutant type plasmid was as described above with outer sense (5'-3') GGTTTCAGATACAGTTGTAG (position 1013-1031) and antisense (5'-3') CAAATGATTTAAGTCTCCG (position 1356-1374) and inside mutagenesis primers, sense (5'3') CGGACGAAACATACTGCATAGATAATG, antisense (5'-3') CATTATCTATGCAGTATGTTTCGTCCG (position 1277-1301). The annealing temperature for the first PCR was 54°C and 50°C for the second PCR. The fragments obtained with the desired mutation for both parasites were purified, cloned and subsequently sequenced.

A.2.5. Genotyping by the Pyrosequencing Method

Pyrosequencing was used to detect a possible SNP in the genomic DNA from the field samples. First, a smaller fragment of DNA from the control plasmids that surrounded the position 200 was amplified. Subsequently, we amplified the same portion of β-tubulin DNA of eggs isolated from individual *A. lumbricoides* adult worms from Kenya, Zanzibar, Uganda and DNA from pools of eggs from Panama and Kenya. For *T. trichiura*, DNA of individual worms from Kenya and pooled eggs from Panama was also amplified. A fragment of 158 bp of *A. lumbricoides* β-tubulin DNA was amplified with primers: sense (5'-3') AGGTTTCTGATGTGGTGTTGGA and antisense (5'-3') TATGTGGGATTTGTAAGCTTCAG. For *T. trichiura*, a fragment of 163 bp was amplified with gene-specific primers: sense (5'-3') AGGTTTCAGATACAGTTGTAG (position 1211-1231), antisense (5'-3') CAAATGATTTAAGTCTCCG (position 1356-1374). The antisense primer was biotinylated (Invitrogen™, Life technologies, Burlington, ON) at its 5' end. For both reactions, the thermal cycling conditions included an initial incubation at 94°C for 3 min, followed by 50 cycles of 94°C for 45 s, an annealing temperature of 58.7°C for *A. lumbricoides*, 55°C for *T. trichiura*, 68°C for 1 min and a final extension at 68° C for 6 min. Biotinylated PCR products were immobilized on streptavidin-coated Sepharose™ beads (Amersham Biosciences, Piscataway, NJ) and sequencing primers used for SNP analysis in the PSQ™96MA instrument (Biotage™ AB, Charlottesville, VA) were: (5'-3')

GAGAACACGGACGAAACAT (position 1270-1288) for *T. trichiura* and (5'-3') GAGAACACCGATGAAACCT for *A. lumbricoides*.

A.2.6. Confirmation of Genotype Sequences

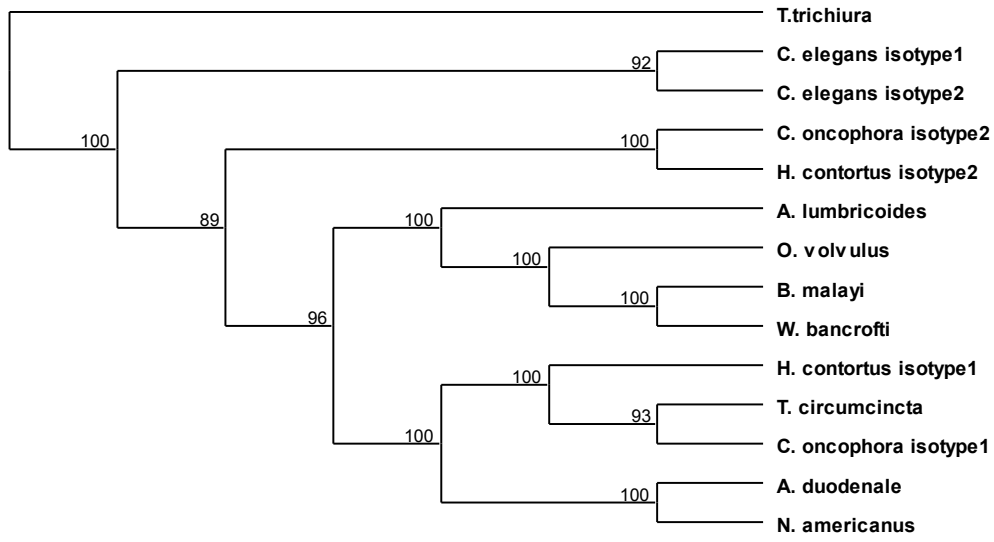
Genotype sequences obtained by pyrosequencing were confirmed by conventional sequencing at the Quebec/McGill University Genome Centre.

A.3. Results

A.3.1. *A. lumbricoides* β-tubulin cDNA Sequence

Fresh RNA from *A. lumbricoides,* naïve for BZ-treatment, allowed us to generate a high quality partial length β-tubulin cDNA (GenBank, EU814697). The length of the portion sequenced was 1137 pb. The translation product revealed a putative sequence of 378 amino acids. The phylogenetic tree (Figure A1.1) showed that the *A. lumbricoides* sequence did not have a close relationship with the Strongylida order which includes the hookworms, *A. duodenale* and *N. americanus* as well as the veterinary nematodes *H. contortus* and *T. circumcincta.* It was also the case for the Trichocephalida which includes *T. trichiura*. In fact, the *A. lumbricoides* cDNA sequence seemed to be more closely related to the Spirudida order which includes the filarial nematodes.

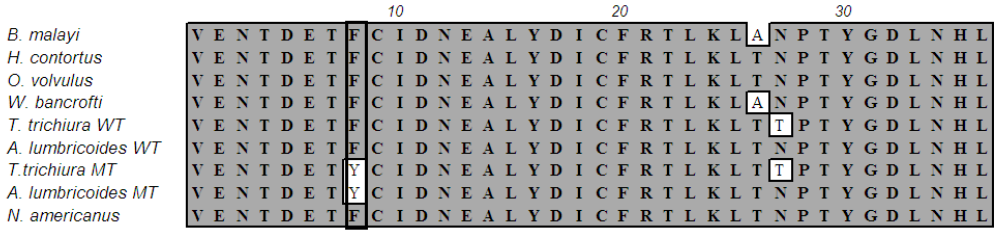
Figure A. 1. UPGMA tree showing the relationship between the β-tubulin cDNA sequence of *A. lumbricoides* with β-tubulin cDNA sequences of other nematodes



A.3.2. Design of diagnostic tests

Based on *A. lumbricoides* and *T. trichiura* β-tubulin, diagnostic SNP assays, optimized with control plasmids, were applied. An alignment of the portion around the codon of interest in the translated β-tubulin protein sequences of wild type (WT) and the mutant type (MT) plasmids with sequences of other nematodes highlighted a high conservation of the protein sequence. It also showed the (T → A) substitution (Phe200Tyr at the amino acid level, Figure A.2) that is associated with BZ resistance in veterinary nematodes [13].

Figure A. 2. Alignment of *Ascaris lumbricoides* and *Trichuris trichiura* β-tubulin protein around codon 200, with related nematodes. WT= wild type, MT= mutant type. The square represents the amino acid at the position of interest.



A.3.3. β-tubulin SNP Genotyping in Parasites from Untreated Subjects

Pyrosequencing assays were designed to genotype the single nucleotide polymorphism (SNP) at codon 200 of β-tubulin. This technique allows sequencing of short fragments of DNA in a very short period of time. The pyrosequencer used can process 96 samples in one hour, and the technique is highly accurate, making the results reliable and easy to interpret. This test was first evaluated with the help of the control plasmids. For *A. lumbricoides* and *T. trichiura,* we obtained distinct pyrogram profiles with the WT and MT plasmids. Pyrograms of each *Ascaris* and *Trichuris* control plasmid showed a single peak for the WT and MT plasmid that identified the 'susceptible' TTC codon and the 'resistant' TAC, respectively, in the codon 200 position. Thus, these results confirmed that the diagnostic test was efficient and could easily and clearly identify the genotype of *A. lumbricoides* and *T. trichiura* worms for this position in the β-tubulin gene.

Once we obtained the expected genotype profiles, we applied the pyrosequencing diagnostic assay to *A. lumbricoides* adult worms and pooled eggs, and to *T. trichiura* adult worms from Kenya from people who had not received treatment with BZ drugs. For *A. lumbricoides* all individual females, males and pools of eggs were "sensitive" T/T. In contrast, we found more diversity in the *T. trichiura* population with regard to the (TAC) SNP. The frequency of heterozygote and homozygote worms was almost the same for male and female worms. Out of 20 males, 11 were homozygous T/T (55%), 8 were heterozygous T/A (40%) and 1 was homozygous A/A (5%). Among the 19 female worms, 11 were homozygous T/T (58%), and 8 were heterozygous T/A (42%).

A.3.4. β-tubulin Genotyping in Parasites Following Mass Drug Administration

In order to investigate if repeated exposure to BZ treatment had an impact on the polymorphism in β-tubulin at codon 200, associated with resistance in veterinary nematodes, we screened the β-tubulin genes of *A. lumbricoides* and *T. trichiura* from different areas where mass drug administration (MDA) programs had been in operation . Pyrosequencing assays, designed previously for each species were applied to detect the presence or absence of TTC or TAC at the SNP of interest. DNA from pooled eggs or DNA from individual worms was analysed. TTC and TAC at codon 200, in the parasite samples, were confirmed by conventional sequencing conducted at the Quebec/McGill University Genome Centre.

The genotype frequencies obtained for all species are shown in and are as follows:

- For *A. lumbricoides* from Uganda and Zanzibar all individual worms were T/T (100%)
- For *A. lumbricoides* from Panama, all the egg pools genotyped were only T (100%)
- For egg pools of *T. trichiura* from Panama, T and A, or A only, were found but no T only was found:

- Out of 8 samples, 3 were T plus A (37%), and 5 were A only (63%).

A.4. Discussion

The possible development of resistance to ALB and MBZ in human nematodes is a threat to MDA programs [8]. A number of studies have reported a reduced efficacy of ALB and MBZ anthelmintics in human STH after repeated treatments [9,14]. Resistance in other nematodes is known to be associated with mutation in β-tubulin preventing the binding of BZ drugs [15]. Consequently, there is a need to investigate and monitor the frequency of SNPs in the β-tubulin gene [16,17] that have been associated with BZ resistance. Bennett and co-workers used conventional sequencing on 72 individual *T. trichiura*, mostly from untreated individuals, and did not observe polymorphism at codon 200 in β-tubulin [18]. Using real time PCR and pyrosequencing, Schwab and colleagues have developed rapid genetic assays for individual *Wuchereria bancrofti* microfilaria [19]. Schwenkenbecher and co-workers have used real time PCR to assay for resistance SNPs in hookworms [20], but did not report finding the resistance associated SNP. However, the β-tubulin gene of *A. lumbricoides* had

not been previously analysed. In this study, we developed pyrosequencing assays to detect SNPs in the β-tubulin genes of *A. lumbricoides* and *T. trichiura*, and analyzed samples obtained from naïve or benzimidazole treated individuals. Control plasmids, WT (TTC) and MT (TAC) were analyzed using the pyrosequencing method. The expected sequences, TTC ("sensitive") and TAC ("resistant") were obtained indicating that the assay was efficient. The development of these genetic assays allows a rapid screening method for the detection of possible resistance alleles in human parasites. This development is consistent with the aim of the Consortium for Anthelminthic Resistance SNPs (CARS) to develop panels of molecular markers for anthelmintic resistance in human and veterinary nematodes [15]. To date, there are not many reliable and accurate tools for diagnosing resistance in STH. Biological tests are used to assess resistance [21,22] but have limited application as they generally can detect resistance only if the proportion of resistant worms is more than 25% [23]. The development of molecular assays for SNPs in the β-tubulin genes of STH gives new hope for monitoring for anthelmintic resistance. This is particularly important considering the difficulty in measuring resistance in helminth parasites of humans compared to helminth parasites of animals [24].

We genotyped STH species from Kenya before the implementation of MDA with ALB or MBZ in order to get baseline information on the SNP frequencies in the β-tubulin genes. In *A. lumbricoides* samples, heterozygous TTC/TAC or homozygous TAC/TAC were not found in samples from non-treated subjects. However, it would be interesting to do more sampling to confirm that the

homozygous "sensitive" genotype predominates in different *A. lumbricoides* populations. It would also be interesting to analyse parasites from the same population of hosts after several rounds of treatment with ALB to see if repeated treatment over some years would result in the Tyr200 SNP being detected in *A. lumbricoides* worms.

In contrast, in *T. trichiura*, the SNP with TAC at codon 200 of β-tubulin was present in samples from the same Kenyan population. In *T. trichiura*, only one β-tubulin gene has been identified even with low stringency Southern blots [11]. This suggests that *T. trichiura* may carry only one isotype of the β-tubulin and that molecular change in this β-tubulin alone might result in resistance. In some veterinary nematodes, the sensitivity or resistance to BZ can be modulated by a second β-tubulin isotype [25]. Samples with both TTC and TAC, as well as others with TAC alone were identified in the *T. trichiura* population. Nevertheless, our sample size was low: out of 39 *T. trichiura* individual worms, 18 were heterozygous TTC/TAC and one individual was homozygous TAC/TAC. However, these frequencies may not be representative of a wider population of *T. trichiura*. Taking into account that the study was carried out in an area where MDA with BZs had not previously been implemented, the finding of a moderately high frequency of heterozygotes, as well as a low frequency of the "resistance" homozygote genotype, raises a concern should BZ deworming programs be implemented in the same region. However, even though the SNP was found as heterozygous in many parasites, a resistance phenotypic may not be apparent as the resistance may be recessive as has been reported for the Phe200Tyr SNP

associated with resistance in *Teladorsagia circumcincta* [26]. This may delay the appearance of a resistance phenotype. However, further work will be necessary to correlate susceptibility/resistance phenotypes and genotypes to confirm the role of the codon 200 SNP with resistance in *T. trichiura* and to determine whether resistance is recessive, semi-dominant or dominant. However, repeated exposure with multiple rounds of treatment could lead to the loss of the susceptible allele as has already been demonstrated in veterinary nematodes [8,27].

Even though the "resistance" (TAC) SNP was found in *T. trichiura* from MDA-naïve subjects, it is important to take into consideration that deworming programs are already common and have existed for many years in other regions of Kenya [28-30]. Communities or regions targeted are quite close to each other and people from treated areas could easily travel to non-treated areas [31].

It is interesting to note that many studies have demonstrated the lower efficacy of BZ drugs against *T. trichiura* compared with *A. lumbricoides* [9,32,33]. However, the factors involved in this difference of efficacy are not known, but could include pharmacokinetics, given the different locations of *A. lumbricoides* and *T. trichiura*, or differences in genetic predisposition to BZ susceptibility. Our current results could support the idea that the difference in sensitivity, between these species, could be due to the occurrence of different alleles with alternatively TTC or TAC at codon 200 in the β-tubulin gene within the gene pool of *T. trichiura*. In veterinary nematodes a number of factors including the frequency of anthelmintic use, proportion of the parasite population exposed to treatment, parasite turnover and other factors may contribute to the

rate of development of resistance [4]. Implementation of very large scale control programs for STH and lymphatic filaria could increase drug selection pressure and possibly the frequency of the Tyr200 SNP in *T. trichiura*. Further analyses on the Phe200Tyr SNP in β-tubulin of *T. trichiura* worms and correlation of its frequency with benzimidazole efficacy will be important to determine so that the possible development of drug resistance as part of MDA programs for STH could be monitored by these assays. If the Tyr200 SNP is confirmed to be associated with BZ resistance in *T. trichiura*, it will also be important to investigate aspects of population dynamics which impact on the rate of change in SNP frequency in populations under drug pressure.

The final objective of our study was to determine if the frequency of the β-tubulin SNP varies after repeated treatment with BZ and if the resistance-associated SNP was high in *A. lumbricoides* and *T. trichiura* samples from areas where there had been MDA with ALB or MBZ. None of the *A. lumbricoides* samples from Panama, Uganda or Zanzibar, examined by pyrosequencing, carried the TAC mutation at codon 200 in the β-tubulin gene. However, in pooled egg samples of *T. trichiura* from Panama, we found the Phe200Tyr SNP in egg pools from hosts who were reported to have been treated with ALB. For the *T. trichiura* pooled egg samples from Panama, both mixed TTC/TAC and TAC alone, in different pools, were found. It is important to point out that as we used pooled eggs, we could not determine the frequency of different genotypes in the worm population. This means that SNP frequencies refer to between *T. trichiura* egg pools and not within a particular egg pool. Because of this, and the small number

of samples, the SNP frequencies must be interpreted with caution. Based on the experience in veterinary nematodes where benzimidazole resistance appears to be recessive [26], a high frequency of the homozygous "resistance" genotype could affect the cure rate and the drug efficacy, and repeated treatment may increase the frequency of homozygous "resistance" genotypes and lead to a rapid development of drug resistance. A study carried out in South Africa on the drug efficacy of 400 mg ALB demonstrated a low cure rate for *T. trichiura* and the authors concluded that this drug was not appropriate for a deworming program in this region [34]. There is an urgent need for studies correlating drug efficacy with genotype.

Knowledge of the β-tubulin sequences will enable us to develop similar pyrosequencing assays for alternative SNPs in β-tubulin of STH, at codons 167 and 198, known to be involved in drug resistance in cyathostome nematodes [26,35] and occasionally in *H. contortus* [7,36].

A.4.1. Conclusion

The effectiveness of broad-spectrum anthelmintic drugs has been proven since their introduction. Examples from veterinary medicine indicate that, unless great care is taken to preserve the effectiveness of these drugs, the days of safe and easy helminth control may become a thing of the past. Studies such as ours are thus required to help control program managers to make appropriate decisions for the design of treatment programs against these harmful parasites. In this study, we described a reliable, fast and easy DNA assay based on a pyrosequencing technique for two soil-transmitted nematodes of humans. This technique allows the SNP analysis of large numbers of egg samples or other parasite stages, in a

short period of time. For the first time, we characterized the partial β-tubulin cDNA and genomic DNA sequence of *A. lumbricoides*. Knowledge of the β-tubulin sequences is important as little is known about resistance to benzimidazole drugs in human helminths and also because of the rapid development of drug resistance in veterinary nematodes. The SNP with TAC was found in individual worms of *T. trichiura* from non-treated people in Kenya, and in *T. trichiura* egg pools from treated people in Panama. These findings provide a possible explanation for the sometimes low efficacy of benzimidazole anthelmintics against *T. trichiura* and an important warning of the possibility that resistance may develop, particularly in *T. trichiura*. It is crucial to continue monitoring for the frequency of the codon 200 TTC/TAC SNP in areas under MDA and to confirm whether the TAC allele confers BZ resistance in these STHs.

A.5. Acknowledgments

It is with deep regret that we record the sudden and tragic death of Richard Suswillo during the course of this study. Richard, a parasitologist much respected for his proficiency in field work, led the collection of the parasite material in East Africa. His commitment, good will and boundless energy are sorely missed by the whole team.

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APPENDIX B

Supplemental Table S1

Table S 1. Primers used for amplification of STHs β-tubulin SNP regions and for pyrosequencing.

Position 167			
STHs	**Sense primer (5'3')**	**Antisense primer (5'-3')**	**Pyrosequencing primer**
A. lumbricoides	TCCGTGAAGAATACCCCGACA (position 47-68)	GCCACACTTGAACCTGCTAACG (position 54-75)	ACCCCGACAGAATCATGAGCTCG (position 59-81)
T. trichiura	GAGTATCCTGACCGAATTATGACA (position 1116-1139)	ACGACGTGAACAGTATCAAACAAC (position 1171-1194)	TGACCGAATTATGACAACT (Position 1124-1148)
Hookworm	GTGACTGTCTCCAGGTAATTCG (position 867-888)	CTATAACGTACCTTTGGCGAGGG (position 1065-1087)	GATAGAATCATGTCCTCGT (Position 1035-1053)
Position 198-200			
STHs	**Sense primer (5'3')**	**Antisense primer (5'-3')**	**Pyrosequencing primer**
A. lumbricoides	AGAGCCACAGTTGGTTTAGATACG (position 318-341)	AGGGTCCTGAAGCAGATGTC (position 489-508) CAGATGTCGTACAAAGCCTCATT (position 476-498)	GGTTGAGAACACCGAT (position 440-455)
T. trichiura	CGCCTTTTTAGGTTTCAGATACA (position 1202-1224)	GTCTCCGTAAGTTGGTGTTGTTAA (position 1339-1362)	GGTAGAGAACACGGACG (Position 1266-1282)
Hookworm	TTTCCGACACTGTGGTTGAG (position 1844-1863)	GAGTTCGTTACTAGCCAGCTCACC (position 2006-2029)	GAGAATACAGATGAGACCT (Position 1110-1128)