Genetic Markers of Benzimidazole Resistance among Human Hookworms (*Necator americanus*) in Kintampo North Municipality, Ghana

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Abstract. Hookworm infection causes anemia, malnutrition, and growth delay, especially in children living in sub-Saharan Africa. The World Health Organization recommends periodic mass drug administration (MDA) of anthelmintics to school-age children (SAC) as a means of reducing morbidity. Recently, questions have been raised about the effectiveness of MDA as a global control strategy for hookworms and other soil-transmitted helminths (STHs). Genomic DNA was extracted from *Necator americanus* hookworm eggs isolated from SAC enrolled in a cross-sectional study of STH epidemiology and deworming response in Kintampo North Municipality, Ghana. A polymerase chain reaction (PCR) assay was then used to identify single-nucleotide polymorphisms (SNPs) associated with benzimidazole resistance within the *N. americanus* β -tubulin gene. Both F167Y and F200Y resistance-associated SNPs were detected in hookworm samples from infected study subjects. Furthermore, the ratios of resistant to wild-type SNP at these two loci were increased in posttreatment samples from subjects who were not cured by albendazole, suggesting that deworming drug exposure may enrich resistance-associated mutations. A previously unreported association between F200Y and a third resistance-associated SNP, E198A, was identified by sequencing of F200Y amplicons. These data confirm that markers of benzimidazole resistance are circulating among hookworms in central Ghana, with unknown potential to impact the effectiveness and sustainability of chemotherapeutic approaches to disease transmission and control.

INTRODUCTION

Soil-transmitted helminth (STH) infections, primarily caused by hookworms (*Necator americanus*, *Ancylostoma duodenale*, and *Ancylostoma ceylanicum*), *Ascaris lumbricoides*, and *Trichuris trichiura*, are widely prevalent in the tropics, with nearly 2 billion people living in endemic areas.^{1,2} Prevalence and intensity are highest in rural, resource-limited settings, where scarce access to sanitation contributes to transmission. Soil-transmitted helminth infections are rarely fatal, but chronic infection in children is associated with anemia and stunting, as well as potential delays in cognitive development and school performance.^{3,4} Approximately 500 million people worldwide are infected with hookworms, including roughly 120 million in sub-Saharan Africa.^{1,2}

Strategies for control of hookworms and other STHs rely on periodic deworming of groups at greatest risk of morbidity. The World Health Organization (WHO) currently recommends annual or twice-yearly mass drug administration (MDA) of anthelmintics, including mebendazole or albendazole, to school-age children (SAC).⁵ Efforts to scale MDA and reach all vulnerable populations have been bolstered by the commitment of pharmaceutical companies, private foundations, and nongovernmental organizations, which have pledged to distribute more than a billion doses of deworming drugs annually through 2020.

Despite this commitment, the long-term health benefit of MDA has recently been questioned,^{6,7} with additional concerns raised about the potential for genetically mediated parasite resistance emerging following repeated exposure to deworming.^{8,9} This is especially relevant to the

benzimidazoles (albendazole and mebendazole), as repeated use in animals has led to widespread resistance in gastrointestinal parasites.^{9–13} In particular, studies have implicated three single-nucleotide polymorphisms (SNPs) within the coding sequence of the nematode β -tubulin isotype-1 gene, the target of benzimidazole drugs, which confer resistance in veterinary nematodes, including the dog hookworm *Ancylostoma caninum*.^{12–14} These SNPs result in amino acid substitutions at aa167 (TTC > TAC; Phe > Tyr; F167Y), aa198 (GAG > GCG; Glu > Ala; E198A), or aa200 (TTC > TAC; Phe > Tyr; F200Y), which alter the binding of benzimidazoles to the β -tubulin protein.^{15,16} Although studies have identified resistance-associated SNPs in human filarial nematodes,^{17,18} few studies have aimed to define genetically mediated resistance in communities where benzimidazoles are administered for the control of STHs.^{8,19–22}

We report here the use of a molecular approach to detect resistance-associated SNPs in hookworms (*N. americanus*) infecting SAC in the Kintampo North Municipality in central Ghana, complementing prior studies characterizing the prevalence and intensity of infection and response to albendazole.^{23–25} Our findings show that hookworm egg samples in Kintampo contain SNPs that could be associated with benzimidazole resistance. We also show that albendazole treatment is associated with an increase in the proportion of two SNPs (F167Y and F200Y) in parasites collected from subjects not cured by albendazole. In addition, we report a previously unrecognized association between the E198A and F200Y SNPs. Taken together, the data underline the importance of monitoring communities for the impact of genetically mediated resistance on deworming treatment response.

METHODS

Ethics approval and consent to participate. Before initiating these studies, approval was obtained from the Human

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Investigations Committee of Yale University, New Haven, CT, and the institutional review board of Noguchi Memorial Research Institute for Medical Research University of Ghana, Accra Ghana. After obtaining informed adult guardian/parental consent, SAC were assented and invited to respond to a demographic and behavior questionnaire.²³

Sample collection. Hookworm egg samples were collected as part of a 2010 field study in the Kintampo North Municipality, which assessed the impact of host nutrition on hookworm infection status and response to deworming with albendazole.²³ School-aged children (ages 6–11 years; *n* = 279) provided fresh fecal samples, which were analyzed for the presence of STH eggs using Kato–Katz microscopy, according to WHO guidelines.²⁶ Hookworm infection was identified in 109/279 subjects (39%), each of whom was referred to Ghana Health Service staff for directly observed treatment with a single dose (400 mg) of albendazole (GlaxoSmithKline, Middlesex, United Kingdom). Fecal samples were then collected from 107/109 (98%) of treated subjects at 10–14 days after drug administration to assess response.

Control plasmid preparation. Separate plasmids, each containing a single positive control DNA insert, were prepared by cloning PCR amplicons spanning base pairs 789–2417 of the β -tubulin 1 genomic DNA (gDNA) sequence into the pCR2.1 vector using primers P789F and P2147R (Table 1). The plasmid DNA was then used as template for site-directed mutagenesis to introduce a single base change in the codons corresponding to aa167 (TTC > TAC; F167Y) or aa200 (TTC > TAC; F200Y) as previously described.²⁷ Complementary DNA strands, generated using primers containing a single nucleotide change (Table 1), were annealed and used to transform *Escherichia coli*.

Detection of SNPs using quantitative PCR (qPCR). The presence of alleles corresponding to wild-type (WT) and mutant (MT) SNP nucleotides at F167Y and F200Y was determined for *N. americanus* eggs collected from individual study subjects.²³ Genomic DNA was extracted from previously frozen hookworm eggs using the QIAamp DNA stool kit (QIAGEN, Carlsbad, CA), following the manufacturer’s suggested protocol. In separate reactions, gDNA and shared primers (167R1 or 200F1) were combined with either “WT” (167F1 or 200R1) or “MT” (167F2 or 200R2) detection primers (Table 1, Figure 1) and iQSupermix (Bio-Rad, Carlsbad, CA; final concentration 25 U/mL iQtaq DNA polymerase, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each deoxyribonucleotide

FIGURE 1. Single-nucleotide polymorphism (SNP)-specific quantitative PCR. Separate PCRs were used to amplify the wild-type and mutant loci corresponding to aa167 (A) and aa200 (B) codons in the *Necator americanus* β -tubulin isotype-1 gene. In both SNPs, the mutation is in the second nucleotide of the codon triplet, resulting in an amino acid change (TTC > TAC; Phe > Tyr). Amplification reactions proceed only when the 3' terminal nucleotide of the primer anneals to the homologous nucleotide present in the genomic DNA (gDNA), ensuring specificity.²⁸ Using this method, the proportion of SNP alleles was calculated from cycle threshold data as described in Methods.

triphosphate (dNTP) [dATP, dCTP, dGTP, and dTTP], 3 mM MgCl₂, SYBR green I dye, 10 nM fluorescein, and stabilizers). The incorporation of SYBR green into amplification products was detected using a Bio-Rad iCycler iQ real-time PCR detection system. Cycling conditions were 5 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 1 minute at 58°C, and 15 seconds at 75°C during which emitted fluorescence was scanned. Following amplification cycles, melting curve analysis (55–95°C) and readings collected every 30 seconds at 0.5°C increments were used to confirm primer specificity and absence of products in the negative control (no template) reactions. Data across runs were normalized to mean cycle threshold (Ct) values obtained for amplification of products from representative positive control plasmid templates (5 pg). The proportion of each MT allele relative to the proportion of the corresponding WT allele for each gDNA sample purified from pooled eggs collected from single study subjects was calculated using the following equation:^{19,21,22,28,29}

$$\text{The proportion of MT allele} = \frac{1}{(2^{\Delta Ct} + 1)},$$

where $\Delta Ct = (Ct \text{ of MT}) - (Ct \text{ of WT})$.

Analysis of the E198A resistance-associated SNP.

Sanger sequencing was performed by the William Keck Facility at Yale University to validate the qPCR amplification reactions. Observations of the aa198 SNP (GAG > GCG; E198A), present as a peak in chromatogram traces collected from aa200 PCR products, were further characterized using fluorescent trace data (relative fluorescent units [RFUs]) collected for each nucleotide. Data were collected for each locus using the ABI base caller algorithm in the DNA Sequencing Analysis Software version 5.1 (Applied Biosystems, Inc., Foster City, CA). Raw numerical peak height data were then extracted from sequence trace files using the ThermoFisher (Waltham, MA) ab1 Peak Reporter tool (<https://apps.lifetechnologies.com/ab1peakreporter>) and ThermoFisher

TABLE 1

Oligonucleotide primers used for SDM and for the detection of individual SNPs

Primer name	Sequence 5' → 3'
SDM primers	
P789F	GGCGAAGGGGCACTAACAACCTG
P2147R	GCCAACTTACGAAGATCAGCATTCAACTGACC
F167YF	ATCATGTCCTCGTATTCCGTTGTACCC
F167YR	GGGTACAACGGAATACGAGGACATGAT
F200YF	AGAATACAGATGAGACCTACTGTATTG
F200YR	CAATACAGTAGGTCTCATCTGTATTCT
SNP detection primers	
167F1	CCCTGATAGAATCATGTCCTCGTA
167F2	CCCTGATAGAATCATGTCCTCGTT
167R1	GATCTCACCTTGGGTGATGG
200F1	CTACCCCTTTCGTCATCAA
200R1	AGAATACAGATGAGACCTA
200R2	AGAATACAGATGAGACCTT

SDM = site-directed mutagenesis; SNP = single-nucleotide polymorphism.

Cloud QC Quality Check module tools available online.³⁰ Relative fluorescent units were compared as a percentage of total combined fluorescence for all four nucleotides at the aa198 SNP position in aa200 MT ($n = 80$) and aa200 WT ($n = 82$) PCR products.

Statistical analysis. The data are presented as mean proportion of MT alleles present within each gDNA pool. The nonparametric two-tailed Mann–Whitney U test was used to compare the proportion of each SNP allele measured in pre- and posttreatment gDNA samples. A paired Wilcoxon signed-rank test was used to compare pre- and posttreatment SNP frequencies for all individuals with measurable hookworm pre- and posttreatment. Bivariate correlations were used to explore relationships between the proportion of individual SNP alleles and response to albendazole, as measured by egg reduction rate. Regression analysis was then used to further assess identified relationships using Stata 14.0 (Stata Corp, College Station, TX).

RESULTS

Detection of resistance-associated SNPs in *N. americanus*.

In 2010, 286 SAC residing in eight villages within the Kintampo North Municipality of Ghana were enrolled in an epidemiological investigation of nutritional risk factors for hookworm infection and response to deworming treatment.²⁴ Using samples collected from enrolled children, fecal microscopy revealed a 39.1% (109/279) prevalence of hookworm in the absence of *Ascaris* or *Trichuris* infections. The cure rate after a single dose of albendazole (400 mg) was 43% (46/107), and the mean fecal egg count reduction rate was 87%. As previously reported,²³ 94/103 (92.1%) were positive for *N. americanus* by PCR and two samples contained both *N. americanus* and *A. duodenale*.

Two resistance-associated SNPs (F167Y and F200Y) were detected in both pre- and posttreatment *N. americanus* samples collected from study subjects in Kintampo. As shown in Figure 2, the proportion of allele F167Y was $1.7 \pm 0.3\%$ in pretreatment samples ($n = 57$), with a 3-fold increase to $5.0 \pm 0.6\%$ measured in posttreatment, microscopy positive samples ($n = 34$; $P < 0.0001$). The F200Y resistance-associated SNP was more common than F167Y, with a mean allele proportion of $41 \pm 3\%$ among pretreatment samples ($n = 69$). Treatment trended toward enrichment of F200Y, with an increase in mean allele proportion of 11% ($41 \pm 3\%$ – $52 \pm 5\%$) among posttreatment samples ($n = 35$), although this result did not reach statistical significance ($P = 0.05$). Additional analysis, limited to individuals in whom matched pre- and posttreatment (microscopy positive) samples were available for testing, showed similar results: frequency of F167Y was significantly higher posttreatment ($P < 0.01$; $n = 27$) and frequency of F200Y was not significantly higher posttreatment ($P = 0.31$; $n = 15$).

Analysis of the E198A resistance-associated SNP. The review of Sanger sequencing trace data for 82 WT and 80 MT PCR products revealed the presence of a third resistance-associated SNP, E198A.³¹ Using fluorescent emissions intensity data (RFUs) for each nucleotide within the aa198 locus, the resistance-associated SNP (GCA > GAA; E198A) was detected almost exclusively within F200Y/MT amplicons, with little representation found in F200Y/WT amplicons (Figure 3). Converted trace data for MT (F200Y)-specific PCR products showed a high degree of heterogeneity in the second nucleotide position of the aa198 codon, with the WT nucleotide

FIGURE 2. Calculated proportion of mutant (MT) single-nucleotide polymorphism (SNP) for F167Y (top) and F200Y (bottom) in *Necator americanus* egg samples collected from individual study subjects before (PRE) and after (POST) treatment with albendazole. Horizontal lines represent the mean MT SNP for each set of samples. The mean values are higher in POST treatment samples compared with PRE treatment samples for F167Y ($1.7 \pm 0.3\%$ PRE vs. $5.0 \pm 0.6\%$ POST; $P < 0.0001$) and F200Y ($41 \pm 3\%$ PRE vs. $52 \pm 5\%$ POST; $P = 0.05$).

(GAG) comprising 66%, the MT site (GCG) 29%, and the remaining two nucleotides contributing roughly 5% of the total RFUs at that nucleotide position. By contrast, among PCR products amplified using the flanking WT aa200 primers, 93% of the total RFUs were also WT at the aa198 genotype (GAG; glutamic acid). Values were unchanged among pretreatment and posttreatment samples, suggesting that exposure to albendazole does not enrich for the co-segregation of the two resistance-associated SNPs.

DISCUSSION

We report here evidence that β -tubulin isotype-1 gene SNPs associated with benzimidazole resistance are circulating among human hookworms (*N. americanus*) in central Ghana. In addition, we show that deworming drug exposure may be associated with an increase in the proportions of these alleles. Combined, these findings highlight the need for monitoring endemic communities for the emergence of genetically mediated benzimidazole resistance.

This study establishes a baseline value of the proportion of benzimidazole resistance-associated SNPs in *N. americanus* hookworms from West Africa. The data show that resistance markers have been circulating in Ghana as early as 2010,

FIGURE 3. Modified scatter plot of nucleotide-specific relative fluorescent units (RFUs) as a share of total fluorescence at the aa198 single-nucleotide polymorphism (SNP) locus, displaying median (horizontal lines), interquartile range (box), and 10th and 90th percentiles (error bars). PCR amplicons corresponding to wild-type (WT) or mutant (MT) aa200 codons were subjected to Sanger sequencing using the common primer F200YF1 (Table 1). Numerical relative fluorescent unit (RFU) values for each nucleotide were converted to a percentage of total combined RFUs at each position in aa200 PCR products. Wild-type aa200 products were almost exclusively (98%) WT at the aa198 locus (GAG; glutamic acid). However, MT aa200 products showed heterogeneity at the aa198 locus, with approximately 25% of the RFUs corresponding to the MT E198A SNP (GCG).

complementing prior studies.^{19–22} Importantly, the proportion of two MT SNPs (F167Y and F200Y) increased in hookworm egg samples collected from subjects who continued to harbor adult worms as they were not cured by single-dose albendazole. This result is interesting in view of the prior report that *N. americanus* egg isolates from subjects who were not cured also exhibited decreased susceptibility to albendazole using an *in vitro* egg hatch assay.²³ The data presented here are consistent with this earlier observation, suggesting that albendazole exposure may alter both the phenotype and the genotype associated with drug resistance in human hookworms. The methodology in this study can now be used to characterize temporal and spatial changes in resistance gene profiles in the setting of ongoing MDA with benzimidazoles.

In Ghana, once-yearly deworming of SAC using albendazole for STHs has been ongoing since 2007. Before that time, annual preventive chemotherapy was primarily directed against onchocerciasis (initiated in 1999) and lymphatic filariasis (initiated in 2001). In areas co-endemic for filariasis and STH, albendazole is administered in combination with ivermectin, another broad-spectrum anthelmintic. Separate studies from Ghana have shown reduced effectiveness of ivermectin against *Onchocerca volvulus* following repeated exposure,³² and *Wuchereria bancrofti* worms from communities treated with both albendazole and ivermectin had β -tubulin mutations associated with benzimidazole resistance.¹⁷ The data reported here show that anthelmintic exposure has the potential to increase the proportion of resistance mutations in STHs as well, further highlighting the need for monitoring of treated communities.

One limitation of the methods applied in this study is the use of pooled egg samples as the source of hookworm DNA, precluding detailed genotyping of individual worms, which is the gold standard for defining allele frequency of a particular

SNP within a single organism.³³ However, the Germer et al.²⁸ method of dividing equal aliquots of pooled gDNA into two separate PCRs along with locus-specific primers (Table 1) is a suitable and cost-effective alternative for determining the relative proportion of each SNP (WT versus MT) within a pool of hookworm eggs prepared from an individual fecal sample. The relative difference in PCR cycles required to amplify each allele is proportional to the amount of each SNP and independent of the total amount of gDNA present. The PCR method described here is especially well suited for comparing the ratio of WT to MT loci in eggs excreted by individuals within communities following drug treatment and for comparing levels of resistance-associated SNPs across time. Because the method accounts for the heterogeneity between and within populations, it is possible to screen large numbers of subjects using a PCR method and draw comparisons. Detailed analysis of allele frequencies and detection of homozygosity would require assessment of individual adult worms, eggs, or larvae; such an approach is highly labor intensive, costly, and may not provide additional insight into the differences between infected people or endemic communities, or changes attributable to drug exposure. It is also possible that the use of a deep sequencing technique might have provided more robust assessment of the relative proportions of WT versus MT SNPs. However, the prohibitive cost of such analysis, coupled with the limited amount of DNA available from each sample extraction, provided the rationale for choosing the SNP-specific PCR method presented here. Present studies are underway comparing deep sequencing methods with PCR from samples collected in Kintampo and other endemic communities.

A second limitation of this study is the lack of a statistically significant association between cure rate and/or egg reduction rate and the proportions of F167Y or F200Y SNPs. This could be due to several possibilities. First, the clinical response to deworming is influenced not only solely by parasite factors, but also by host factors, including the timing of drug administration and host nutritional status.²⁵ Second, the manifestation of genetically mediated benzimidazole resistance in *N. americanus* may require multiple mutations, as seen in veterinary helminths.^{14,34} It is also possible that the accuracy of the PCR method used was reduced because of partial degradation of DNA samples during storage before analysis.

We detected an unexpected association between the E198A and F200Y SNPs by interrogating sequence data from amplicons targeting the aa200 locus, which showed that the E198A resistance marker was present in greater proportions in samples also containing the F200Y SNP. To our knowledge, such an association or linkage between two presumably independent resistance genes has not previously been reported for a human STH. Further research is needed to define the role of these SNPs in mediating benzimidazole response in *N. americanus* and other hookworms.

Annual or twice-yearly MDA with anthelmintics is currently recommended by WHO for control of morbidity associated with STH infection in SAC based on the favorable cost, excellent safety profile, and acceptable activity against multiple helminths.⁵ However, despite recent commitments to scale up MDA coverage, controlled deworming trials in endemic communities have shown inconsistent clinical, social, and economic benefit.^{6,7,35,36} Potential explanations for the disparate

results of deworming treatment include the variable efficacy of single-dose regimens of benzimidazole anthelmintics against STHs, especially *Trichuris* and hookworms.^{24,37,38} In addition, the WHO strategy of school-based deworming excludes treatment of significant reservoirs of infection and transmission within endemic communities, specifically adults, pre-SAC, and children who do not regularly attend school.^{39,40} Finally, reinfection often occurs quickly,^{41,42} so that MDA may need to be repeated more frequently than current recommendations to achieve long-term and sustainable benefits.

Among the concerns raised about expanding MDA is the potential emergence of genetically mediated resistance to benzimidazoles, a phenomenon that has been well described in veterinary nematodes.^{9,31} Benzimidazoles inhibit microtubule assembly by binding to tubulin and preventing polymerization,¹⁵ and point mutations within the β -tubulin isotype-1 gene result in amino acid substitutions that alter protein structure and reduce drug binding.¹⁶ Most notably, the substitution of phenylalanine to tyrosine at aa200 (F200Y) and aa167 (F167Y), and the substitution of glutamic acid to alanine at aa198 (E198A) have been implicated as resistance-associated SNPs in animal and plant nematodes.^{31,43,44} Interestingly, repeated use of the macrocyclic lactone moxidectin selects for the F167Y and F200Y substitutions in the ruminant nematode *Haemonchus contortus*. Although a similar phenomenon has not been described in human nematode parasites, this observation raises the possibility that exposure to non-benzimidazoles may promote cross-resistance to this class of anthelmintic.⁴⁴ This is especially relevant in sub-Saharan Africa, where combined MDA of albendazole with ivermectin to control filariasis could potentially exert additive selective pressure on hookworms and other STHs.

Although reduced effectiveness of frequently used anthelmintics for hookworms has been observed,^{23,24,37} research aimed at identifying genetic markers of benzimidazole resistance in human isolates has not demonstrated a correlation between genotype and clinical response to deworming.^{19–22} Two studies reported low proportions of the F167Y and F200Y SNPs in *N. americanus* samples (adult worms and pooled third-stage larvae) collected from children on Pemba Island, Tanzania, who had received multiple rounds of mebendazole.^{19,22} More recent data from Pemba confirmed a low proportion of the F200Y SNP (approximately 10%) in pooled larvae, whereas samples collected from Haiti showed the mutation was present at an overall allele proportion of 36% in 27 pooled egg samples.²¹ A similar study of hookworm eggs collected in Kenya, Haiti, and Panama²⁰ reported that resistance mutations were rare at baseline and that anthelmintic treatment was not associated with an increase in the resistance-associated SNPs.

WHO recommends assessment of anthelmintic drug efficacy in areas where preventive chemotherapy has been in place for 4+ years and in settings of reduced treatment effectiveness.²⁶ Field studies in Ghana^{23–25} demonstrate a sustained prevalence of hookworm infection and variable effectiveness of single-dose albendazole. In combination with the molecular data presented here, we suggest more frequent monitoring of hookworm treatment response at the individual and community levels in Ghana, including PCR assessment of genetic markers of resistance. Importantly, further research is needed to determine the potential impact of genetically mediated resistance on the effectiveness of global hookworm control strategies.

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