Absence of *β*-tubulin SNPs associated with drug resistance in *Ascaris lumbricoides* infections of school-age children in Bungoma County, Kenya

ABSTRACT

Aims: This study compared the prevalence of *Ascaris lumbricoides* infections pre- and post-deworming in school-age children in Bungoma County, western Kenya with a view to detecting single nucleotide polymorphisms (SNPs) associated with drug resistance in these infections.

Study design: A longitudinal study design was adopted.

Place and Duration of Study: The study was conducted in Bungoma County in the western Kenya. Stool samples were collected one month before (October 2021) and two weeks after the deworming exercise (January 2022).

Methodology: 414 school-aged children were recruited for the study. A total of 202 stool samples were collected pre-mass drug administration (MDA) while 212 stool samples were obtained post-MDA. Prevalence and infection intensities were determined using the Kato-Katz technique. Genomic DNA was extracted from the pre and post-deworming positive *A. lumbricoides* eggs. Standard PCR generated 564bp amplicons surrounding the target codons 167, 198, and 200 of the *β*-tubulin gene of *A.lumbricoides* which have been associated with the drug resistant phenotype. The amplicons were sequenced using Sanger sequencing to detect the presence of SNPs at these loci.

Results: The overall pre-MDA and post-MDA STH prevalences were 33% and 6% respectively whereas the prevalence of *A. lumbricoides* infections alone was 31% and 4% respectively. *A. lumbricoides* was the dominant infecting helminth species. However, a few samples (<5) had hookworms and *T. trichiura*. Sixteen sequences from were generated covering codons 167, 198 & 200 of the *β*-tubulin gene of *A. lumbricoides*. Sequencing of the DNA samples revealed that no β-tubulin SNPs were present in the *A. lumbricoides* infections.

Conclusion: Persistence of some *A. lumbricoides* infections following MDA using mebendazole is suggestive of the drug resistant phenotype, but other possible explanations need to be ruled out. It is noteworthy that even after over 10 years of use of benzimidazole drugs in the school-based deworming program, there is no evidence of drug resistance SNPs. This study supports the continued use of the current anti-helmintic drugs in this setting. The use of molecular tools for regular surveillance is important to ensure effectiveness of the deworming program.

Keywords: Soil-transmitted helminths, Mebendazole, benzimidazoles, drug-resistance, Mass drug administration, single nucleotide polymorphisms, β-tubulin, Ascaris lumbricoides.

1. INTRODUCTION

Soil-transmitted helminths (STH) is a collective term referring to nematode infections that are transmitted to man through contact with soil contaminated with the eggs or larval stages of these nematode species[1]. They include the small intestinal roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*), and the hookworms (*Necator americanus* and *Ancylostoma duodenale*) [2, 3, 4]. STH

infections are most prevalent in tropical and subtropical regions of the world with the highest prevalence being recorded in sub-Saharan Africa and South-East Asia [5, 6]. Approximately 1.5 billion people across the globe are infected with either hookworms, roundworms, or whipworms[2]. Although STH infections may occur in different demographic groups, the adverse effects of these infections are greatest among children resulting in malnutrition, anemia, stunted growth and impaired cognitive development [2, 7]. In addition, STHs have resulted cumulatively to over 3.5 million disability-adjusted life years (DALYs)[2]. *Ascaris lumbricoides* infections account for the highest morbidity among the STHs [8, 9, 10]. STHs are part of the 20 neglected tropical diseases (NTDS) identified by the WHO as priority diseases for enhanced control and elimination in the recently published road map for NTD 2021-2030[11].

With substantial support from WHO and various stakeholders, increased resources have been directed towards community-based programs and research activities geared to better understand, control, and eliminate STHs. Interventions include improved case management, health education, and WASH programs which are aimed at enhancing sanitation and hygiene practices in high risk communities [2, 10]. The core strategic intervention for the control of STHs is preventive chemotherapy targeting pre-school age children, school-age children (SAC), and women of reproductive age in endemic areas [2]. This deworming intervention aims at ensuring 75% coverage of targeted at risk populations. High coverage is achieved following administration of the medications twice per year in areas where STH prevalence is equal to or greater than 50% and provision of a single administration per year in regions where STH prevalence is between 20% and 50% [2, 12]. In Kenya STH control efforts have been primarily driven through the school-based deworming program targeting SAC and coordinated by the Ministry of Education (MoE) and the Ministry of Health (MoH) with support from partners [13]. Albendazole (ABZ) (400mg) and mebendazole (MBZ) (500mg) are the recommended medications used in MDA programs due to their efficacy (when used as a single dose), affordability and ease of administration [14, 15]. The two drugs belong to the benzimidazole (BZ) group of compounds which work by targeting *β*-tubulin protein monomers which ordinarily occur as dimers in association with α-tubulin monomers that polymerize to form microtubules. The binding of these drugs to *β*-tubulin inhibits microtubule polymerization [16, 17]. This interferes with the structural and cellular functions of the microtubules leading to disruption of cellular transport, intracellular support, and DNA segregation eventually resulting in the death of the parasite [16, 17]

The continued and widespread use of medications is known to create selection pressure for drug-resistant pathogens. This has been observed among bacteria, viruses, protozoans, and veterinary nematodes. There is a growing concern about the possible emergence of BZ-resistant STH parasite strains which might erode the gains achieved by MDA programs. These concerns are valid given that drug resistance species have been identified among veterinary nematodes such as *Haemonchus contortus* where resistance to BZ occurs due to SNPs within the *β*-tubulin gene at codon positions 167, 198, and 200 [18,19] . A study conducted in Kenya, Haiti and Panama in 2013 on human STHs have similarly demonstrated the existence of SNPs in the *A. lumbricoides β*-tubulin gene at codon 167 [15]. The study further demonstrated the existence of SNPs in *T. trichura* at codon 200 which was consistent with an earlier study conducted in Kenya in 2009 [20]. These studies indicated that emergence of drug resistant is a valid concern. The widespread occurrence of such drug-resistant STH species would be a devastating setback in the control and elimination efforts against these helminth infections. Early identification and tracking the extent of the spread of such parasites would be critical to STH control and elimination efforts in endemic areas [21]. Molecular techniques are essential for the rapid and accurate detection of such drug-resistant parasites early enough to mitigate the harmful impact of their widespread distribution [22].

Kenya is listed by the WHO as one of the countries that continues to require antihelmintic MDA [2]. As a result, school-age children are given a single dose of MBZ or ABZ once per year in the in STH-

endemic regions. This study aimed at comparing the prevalence of STH infections before and after mass deworming with MBZ and to determine whether *β*-tubulin SNPs are present in *A. lumbricoides* in the infections that persisted post-treatment.

2. MATERIALS AND METHODS

Study site

This longitudinal study was undertaken in Bungoma County, western Kenya. This county has a 7.3% prevalence of STH infections according to previous research conducted in 2020[13], and has been through multiple rounds of MDA targeting SAC over the last ten years. The study samples were collected from two different schools (Bukirimo RC Primary School and Chongeywo Primary School) one month pre-MDA and two weeks post-MDA. The two schools were purposively selected in consultation with the Kenya Ministry of Health based on the prevalence data collected in preparation for the MDA exercise. The collection of stool samples was conducted in October 2021 and January 2022.

Study population

School-age children from grade 1 to Class 6 were recruited into the study after obtaining parental consent in the form of a sample opt-out form and children's verbal assent. This was consistent with previous practice in studies conducted in the region [13]. School-level permission was also sought and obtained from the head teachers. With Kenya being listed by the WHO as one of the countries that continues to require antihelmintic MDA, SAC are targeted for MDA and were provided with a single dose mebendazole (MBZ) (500mg).

Stool sample collection

Convenience sampling was used for sample collection. On the day of sample collection every eligible child who met the inclusion criteria was provided with a sample container (polypot) labeled with a specific code assigned to each participant for anonymity and instructed on how to collect approximately 25 grams of their stool sample. When receiving the collected samples, the age and gender of the children were recorded. The samples collected were treated as infectious and placed in a cooler box with ice packs and transported to the nearest Level IV hospital laboratory (Bumula Sub-County Hospital Laboratory for samples from Bukirimo RC Primary School and Chwele Sub-County Hospital Laboratory for samples from Chongweyo Primary School). At the hospitals, the samples were analyzed using the Kato-Katz technique (duplicate slides, 41.7mg template) to determine helminth prevalence.

Data processing and analysis

Data was entered into MS Excel spreadsheet. The prevalence of infection (and associated 95% confidence interval) was determined as a proportion based on the number of those who tested positive out of the total number of participants surveyed. The intensity of the infections (eggs per gram, epg) were also determined using WHO guidelines [23]. The positive samples were transported to the Parasitology Laboratory of the Department of Medical Laboratory Sciences at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi for DNA extraction. The stool samples were preserved at -20^{o}C to preserve the helminth eggs prior to extraction of helminth genomic DNA.

Extraction of helminth genomic DNA

Helminth eggs were isolated from the stool samples using the sodium chloride (NaCl) floatation technique and preserved in 70% ethanol in 1.5ml tubes at room temperature. A 1.5ml Eppendorf tube containing the preserved eggs was centrifuged at 5,000 x g for 2 minutes and the supernatant was

removed by pipetting to remove the ethanol. The pellet was washed in two cycles of adding 1ml Phosphate buffered saline (PBS), vortexed at 3500 x g for 1 minute, and centrifuging at 5,000 x g for 2 minutes. The supernatant was discarded by pipetting and the pellet was resuspended in 500 ul of distilled water for DNA extraction from the eggs using a commercial kit by Meridian Bioscience® (Bioline ISOLATE II Genomic DNA Kit), according to the manufacturer's protocol with some modifications which included: initial step of freezing the eggs in liquid nitrogen for 10 minutes followed by thawing at 100°C for 10 minutes for 5 cycles. Next, 0.1mm Zirconia silica beating beads were added and the sample was vortexed at maximum speed (3500 x g) for 5 minutes to mechanically break the egg shells.

PCR amplification of *β*-tubulin gene

Extracted DNA was amplified to generate an amplicon covering the region where the SNPs at codon positions 167, 198 and 200 in the *β*-tubulin gene of *A. lumbricoides* are located. Standard PCR for *A. lumbricoides* was done with a Forward (5`-CCAGCTGACGCACTCGCTTGG-3`) and reverse (5`-ATGGTTGAGGTCTCCGTATGTG-3`) primers that were specific to the flanking regions as designed by Diawara *et al.,* (2013). The PCR reaction mix was prepared by adding 10x standard reaction buffer, 2.5 µl 10nM dNTPs, 0.5 µl Forward Primers, 0.5µl Reverse primers, 0.125 µl Taq DNA polymerase, 5µl DNA template, and 15.875µl Nuclease free water to make a total volume of 25µl. PCR reactions were performed with the following cycling parameters: 95°C for 5 minutes followed by 30 cycles of 95°C for 45 seconds, 59°C for 45 seconds, and 72°C for 1 minute with a final extension step at 72°C for 5 minutes.

Detection of *β*-tubulin 167, 198 and 200 SNPs

Following PCR, the generated amplicons were purified prior to sequencing using a commercial kit by Meridiam Bioscience® (Bioline ISOLATE II PCR and Gel Kit) according to the manufacturer's protocol. A 20µl aliquot of each amplicon was submitted to the Sequencing, Genotyping and Oligosynthesis platform (Segolip) unit at The International Livestock Research Institute (ILRI), Nairobi, Kenya. Multiple sequence alignment was then carried out online using Clustal Omega and SNP frequencies were reported as a percentage.

3. RESULTS

3.1 Prevalence of STHs before and after MDA

A total of 202 pre-MDA samples were collected from grade 1 to grade 6 children. The SAC age range was 8 to 15 years with a mean age of 10.6 years (SD 1.65). The baseline STH prevalence pre-MDA was 33%, (95% CI: 26.7-39.7), whereas that for *A. lumbricoides* was 31% (95% CI: 24.8-37.6). Two weeks post-MDA exercise where the children were treated with MBZ, 212 stool samples were collected. The age range for the children was 7 to 15 years and mean age was 11.0 years (SD 1.60). The post-MDA STH prevalence after MDA dropped to 6% (95% CI: 2.6-8.8), while that of *A. lumbricoides* dropped to 4% (95%CI: 1.5-7) (Table 1).

Table 1*:* Prevalence of soil-transmitted helminths infections pre- and post-MDA

STH species	Pre-MDA, n (%)	Post-MDA, n (%)
Ascaris lumbricoides	63 (31)	9 (4)
Hookworms	2 (1)	2 (1)
Trichuris trichiura	0 (0)	0 (0)
Ascaris lumbricoides and *Trichuris trichiura*	1 (0.5)	0 (0)
Ascaris lumbricoides and Hookworms	1 (0.5)	1 (0.5)
No STH observed	135 (67)	200 (94)
TOTAL	202 (100)	212 (100)

Following stool sample analysis using the Kato-Katz technique, the intensity of infection (eggs per gram) was also calculated based on the WHO guidelines[23].The majority of infections before and after MDA were of light intensity and there were no cases of heavy infections (Table 2).

Table 2: Infection intensity of the positive samples before and after MDA

STH species	Infection Intensity					
	Pre-MDA, n, (%)			Post-MDA, n, (%)		
	Light[a]	Moderate[b]	Heavy[c]	Light[a]	Moderate[b]	Heavy[c]
A. lumbricoides	43 (68)	20 (32)	0 (0)	6 (67)	3 (33)	0 (0)
T. trichiura	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Hookworms	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)

[a]Light infections: *A. lumbricoides* 1 – 4999 eggs per gram (epg), *T. trichura*: 1 – 999 epg, Hookworms 1 – 1999 epg

[b]Moderate infections: *A. lumbricoides* 5000 – 49,999 epg, *T. trichura* 1000 – 9,999 epg, Hookworms 2,000 – 3,999 epg

[c]Heavy infection: *A. lumbricoides* > 50,000 epg, *T. trichura*: > 10,000 epg, Hookworms > 4,000 epg

3.2 Identification of *β*-tubulin SNPs associated with drug resistance in *Ascaris lumbricoides*

For the 9 samples where *A. lumbricoides* infection persisted post-MDA, eggs were isolated from 8 samples using the sodium chloride floatation technique and genomic DNA was extracted. 1 sample was inadequate and was all used up in the Kato-Katz technique. For comparison, 8 samples were selected from the positive samples pre-MDA, eggs isolated, DNA extracted and sequenced as described above. Prior to DNA extraction using the commercial kit, mechanical breakage of the egg shells was confirmed microscopically (Figure 1). Sixteen sequences were generated covering codons 167, 198 & 200 of the *β*-tubulin gene of *A. lumbricoides*. Aligned results from the Sanger sequencing demonstrated the wild-type *A. lumbricoides* with 0% SNPs frequency. All the three codons targeted (167, 198, and 200) were monomorphic for all the samples (Figure 2)

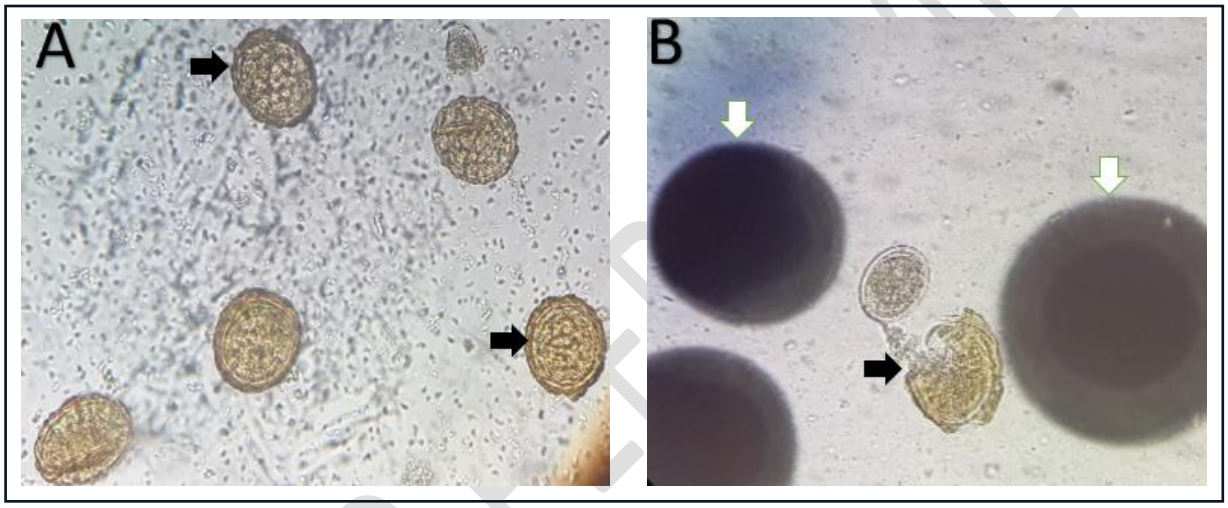

Figure 1: Microscopic analysis of stool samples

(*A) Intact A. lumbricoides eggs (black arrows) before the freeze thawing and bead beating step (B) 0.1mm Zirconia silica beating beads (White arrows), A. Lumbricoides egg with a broken egg shell (black arrow) after the freeze thawing and bead beating step.*

***Figure 2: Multiple sequence alignments of* β-tubulin amplicon**.

The first underlined sequence shows the wild type A. lumricoides (GenBank accession number FJ501301.1) sequences surrounding the target codons 167, 198, and 200 of the β-tubulin gene. Highlighted sequences shows the post-MDA sequences surrounding the target codons 167, 198, and 200 of the β-tubulin gene (BA- Bukirimo Primary school post-MDA, CA- Chongeywo Primary school post-MDA).Un-highlighted shows the pre-MDA sequences surrounding the target codons 167, 198, and 200 of the β-tubulin (CB- Chongeywo Primary school pre-MDA, BB- Bukirimo Primary school pre-MDA).

4 DISCUSSION

In this study, school-age children were targeted and samples were collected pre-MDA and post-MDA with MBZ, with a 33% prevalence pre-MDA. This is a higher prevalence compared to a study done previously in the area where the prevalence was 7.3% [13]. The study further observed that *Ascaris lumbricoides* was the most prevalent STH accounting for 94% of the observed prevalence in the study area, followed by hookworms at 3%, mixed infections of *A. lumbricoides* and *T. trichiura* and *A.lumbricoides* and hookworms accounted for 1.5% of the prevalence each. This is consistent with a study conducted previously in the region which showed that *A. lumbricoides* was the most prevalent STH, followed by hookworms and *T. trichiura* respectively[24]. The findings are also consistent with the global statistics of STH where *A. lumbricoides* accounts for the highest morbidity. On a positive note, however, the study found that of all the positive infections in the area under study, none was a case of heavy infection intensity and a majority of the infections (77%) were light infections, suggesting a positive impact of the 10 years of deworming in this setting. Studies that have been conducted previously where MDA programs have been in place have shown a similar trend where there is a considerable shift of infection from heavy infection intensity towards moderate and light infection intensities [25, 26]. Infection intensity directly relates to the morbidity due to STHs[27]. Few worm burdens (light intensity) cause little or no infection amongst the people infected. High worm burden is associated with a range of symptoms including Intestinal manifestations such as diarrhea, bloating, flatulence, and abdominal pain. In extreme cases, malnutrition, impairment of physical development and growth, general weakness, and malaise may be witnessed[28]. In children, the effects of chronic STHs infections may dramatically affect physical, mental, and educational development. Although worm burden is directly related to children's symptoms,

light infections can also cause significant symptoms in poor community nutritional status [29, 30]. Heavy intensity infections by STHs can cause intestinal obstruction, and the parasites are also associated with chronic malnutrition in children[31] .

Post-MDA where the children were treated with MBZ, there was a significant decline in the overall prevalence from the initial 33% to 6%. These samples were collected two weeks post-deworming exercise which is an optimum timeframe to ensure worm clearance and to rule out re-infection[32]. This trend of reduction in prevalence has been observed previously where the prevalence falls drastically after mass deworming exercise [24]. However, a previous study observed that the prevalence starts rising again after some time which can be attributed to environmental and hygienic conditions or due to parasite strains less susceptible to the drugs [13]. The persistence of the prevalence despite the mass deworming and the fact that the exercise has been going on for the past 10 years may be suggestive of a trend towards resistance associated with SNPS in the *β*-tubulin gene. Just like the infections observed before the MDA, all the infections post-MDA were of light infection intensity (9/12) 75% and moderate infection intensity (3/12) 25%.

The study also checked for mutations in the *β*-tubulin gene for the samples that were positive post-MDA. *B*-tubulin is one of the two protein molecules that join together to make the microtubules, these microtubules are hollow microscopic tubes made up of a tubulin dimer (*β*-tubulin and alpha-tubulin) and form part of the cytoskeleton[31]. The microtubules play key roles in the cell which are cell division, cell movement, and cell transport[33]. *B*-tubulin protein molecules act as the target of the BZ which is a group of compounds used in the treatment STHs and MBZ which is the drug that was used for MDA in this study belongs[34]. These drugs inhibit microtubule polymerization by interfering with cellular structures and functions. This leads to the death of the parasites[16]. Mutations to the *β*-tubulin molecule may therefore confer the parasites with resistance against the BZ[35]. The study analyzed all the sequences obtained from the DNA isolated from positive samples post-MDA and an additional 8 sequences obtained from randomly selected samples pre-MDA. The samples sequenced demonstrated the wild-type *A. lumbricoides* and all the targeted codons were monomorphic for all the samples with 0% SNPs frequency. No *β*-tubulin SNPS associated with drug resistance in STH were identified. A recent study conducted in Honduras demonstrated similar results[36]. It is noteworthy that even after over 10 years of use of benzimidazole drugs in the school-based deworming program, there is no evidence of drug resistance SNPs. This supports the continued use of the current anti-helmintic drugs in this setting. However, a previous study have in contrast identified high-frequency mutations and the *β*-tubulin gene of *A. lumbricoides* especially at codon 167[37]. Different studies on BZA resistance in parasitic nematodes have indicated that this resistance occurs as a result of SNP in the *B*-tubulin gene. In these SNPs, there is a substitution of amino acid phenylalanine by tyrosine which occurs at codons 167 and 200. Likewise, the amino acid alanine replaces glutamate at codon 198. These mutations have on many occasions been associated with drug resistance in STHs [34, 38].

With widespread resistance to BZ in veterinary nematodes having been reported since their first use in the early 1960's and their resistance having been reported in the 1990's[39],[40], it is encouraging to find no such mutations in human nematodes after their usage for several decades having first been used in humans in 1974(MBZ) and 1982 (ABZ)[41,42].

5. CONCLUSION

Persistence of some *A. lumbricoides* infections following MDA using mebendazole is suggestive of the drug resistant phenotype, but other possible explanations need to be ruled out. It is noteworthy that even after over 10 years of use of benzimidazole drugs in the school-based deworming program, there is no evidence of drug resistance SNPs. This study supports the continued use of the current anti-helmintic drugs in this setting. The use of molecular tools for regular surveillance is important to ensure effectiveness of the deworming program.

CONSENT AND ETHICAL APPROVAL

At the county level, approval was sought from the County Health and Education authorities. At school, parental consent was obtained based on passive opt-out consent rather than written opt-in consent due to the routine and low-risk nature of the study procedure which was consistent with the previous researches conducted on STH in SAC in Kenya. In addition, individual assent was obtained from each child before participation in the study. Ethical approval for the study protocol was obtained from the Jomo Kenyatta University of Agriculture and Technology Ethical Review Committee under approval number **JKU/IERC/02316/0411**. A research license for the study was also obtained from NACOSTI under license number **NACOSTI/P/21/14436**.

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