



Absence of β -tubulin SNPs Associated with Drug Resistance in *Ascaris lumbricoides* Infections of School-Age Children in Bungoma County, Kenya

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Authors' contributions

This work was carried out in collaboration among all authors. Author AM conceptualized the study.

Authors PMM, AM and WWA contributed to development of methodology, sample collection and preliminary analysis. Authors AM and MRO supervised the study. Authors AM and PMM conducted data analysis wrote the original draft preparation. Author AM was involved in project administration. All authors read and approved the final manuscript.

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ABSTRACT

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

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Study Design: A longitudinal study design was adopted.

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

Methodology: 414 school-aged children were recruited for the study. Two hundred and two stool samples were collected pre-MDA while 212 were obtained post-MDA. Prevalence rates and infection intensities were determined using the Kato-Katz technique at both time points. Genomic DNA was extracted from selected pre- and all post-deworming positive *A. lumbricoides* eggs. Standard PCR generated a 564bp amplicon that surrounded the target codons 167, 198, and 200 of the *A. lumbricoides* β -tubulin gene which have been associated with the drug-resistant phenotype. Sixteen amplicons were sequenced using Sanger sequencing to detect the presence of SNPs at these loci.

Results: The overall pre-MDA and post-MDA STH prevalence rates 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 were generated covering codons 167, 198, and 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: The persistence of some *A. lumbricoides* infections following MDA using mebendazole suggests the drug-resistant phenotype, but the possibility of less than 100% MDA coverage in schools could be an alternative explanation. However, it is noteworthy that even after over ten years of use of benzimidazole drugs in the Kenya school-based deworming program, there is no evidence of drug resistance SNPs. This study therefore supports the continued use of the current anti-helminthic drugs in this setting. We recommend continued use of molecular tools for regular surveillance of drug-resistant parasites as this is essential to ensure the effectiveness of the deworming program.

Keywords: Soil-transmitted helminthes; 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 transmitted to man through contact with soil contaminated with the eggs or larval stages of these nematode species [1]. They include the tiny 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, with the highest prevalence in sub-Saharan Africa and Southeast 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 most significant among children, resulting in malnutrition, anemia, stunted growth, and impaired cognitive development [2, 7]. In addition, STHs have resulted cumulatively in over 3.5 million disability-adjusted life years (DALYs) [2]. *Ascaris lumbricoides* infections account for the highest morbidity among 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 to understand better, control, and eliminate STHs. Interventions include improved case management, health education, and WASH programs to enhance sanitation and hygiene practices in high-risk communities [2, 10]. The core strategic intervention for controlling 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 binding 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 creates a selection pressure for drug-resistant pathogens. This binding 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 has 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, consistent with an earlier study conducted in Kenya in 2009 [20]. These studies indicated that the emergence of drug resistance 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 of 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].

The WHO lists Kenya 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 STH-endemic regions. This study aimed to compare the prevalence of STH infections before and after the 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

2.1 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.

2.2 Study Population

School-age children from grade 1 to Class 6 were recruited into the study after obtaining parental consent through a sample opt-out form and children's verbal assent. This recruitment 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 is targeted for MDA and was provided with a single dose of Mebendazole (MBZ) (500mg).

2.3 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 collecting 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). The samples were analyzed at the hospitals using the Kato-Katz technique (duplicate slides, 41.7mg template) to determine helminth prevalence.

2.4 Data processing and analysis

Data was entered into an 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) was 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°C to preserve the helminth eggs before extraction of helminth genomic DNA.

2.5 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 five 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.

2.6 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 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 μ l 10nM dNTPs, 0.5 μ l Forward Primers, 0.5 μ l Reverse primers, 0.125 μ l Taq DNA polymerase, 5 μ l DNA template, and 15.875 μ l Nuclease free water to make a total volume of 25 μ l. 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.

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

Following PCR, the generated amplicons were purified before sequencing using a commercial kit by Meridian Bioscience® (Bioline ISOLATE II PCR and Gel Kit) according to the manufacturer's protocol. A 20 μ l 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 conducted online using Clustal Omega, and SNP frequencies were reported as a percentage.

3. RESULTS

3.1 Prevalence of STHs Before and After MDA

Two hundred and two 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 the 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 (n=414)

STH species	Pre-MDA, n (%)	Post-MDA, n (%)
<i>Ascaris lumbricoides</i>	63 (31)	9 (4)
Hookworms	2 (1)	2 (1)
<i>Trichuris trichiura</i>	0 (0)	0 (0)
<i>Ascaris lumbricoides</i> and <i>Trichuris trichiura</i>	1 (0.5)	0 (0)
<i>Ascaris lumbricoides</i> and Hookworms	1 (0.5)	1 (0.5)
No STH observed	135 (67)	200 (94)
All STH	67 (33)	12 (6)
TOTAL	202 (100)	212 (100)

Table 2. Infection intensity of the positive samples before and after MDA (n 414)

STH species	Infection Intensity					
	Pre-MDA, n, (%)			Post-MDA, n, (%)		
	Light ^a	Moderate ^b	Heavy	Light ^a	Moderate ^b	Heavy
<i>A. lumbricoides</i>	43 (68)	20 (32)	0 (0)	6 (67)	3 (33)	0 (0)
<i>T. trichiura</i>	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)

^aLight infections: *A. lumbricoides* 1 – 4999 eggs per gram (epg), *T. trichiura*: 1 – 999 epg, Hookworms 1 – 1999 epg

^bModerate infections: *A. lumbricoides* 5000 – 49,999 epg, *T. trichiura* 1000 – 9,999 epg, Hookworms 2,000 – 3,999 epg

^cHeavy infection: *A. lumbricoides* > 50,000 epg, *T. trichiura*: > 10,000 epg, Hookworms > 4,000 epg

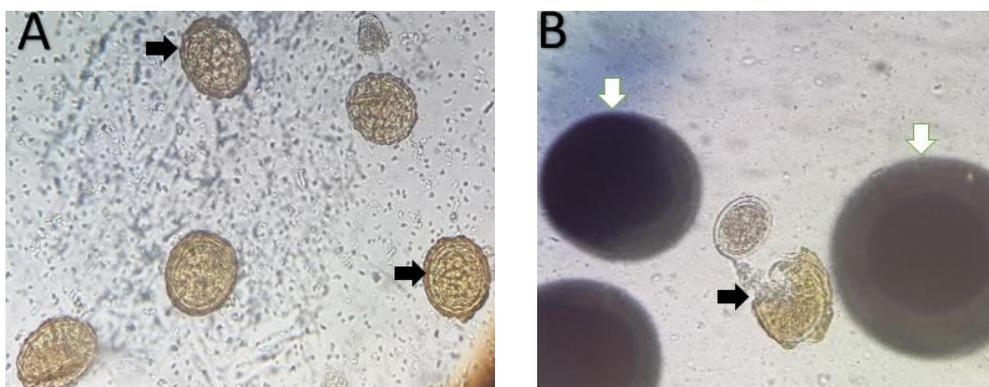


Fig. 1. Microscopic analysis of stool samples

(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

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]. Most infections before and after MDA were light-intensity, with no heavy infections (Table 2).

3.2 Identification of β -tubulin SNPs Associated with Drug Resistance in *Ascaris lumbricoides*

For the nine 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. One sample was inadequate and was all used up in the Kato-Katz technique. For comparison, eight samples were selected from the positive samples pre-MDA, eggs isolated, DNA extracted, and sequenced as described above. Before DNA extraction using the commercial kit, mechanical breakage of the egg shells was confirmed microscopically (Fig. 1). Sixteen sequences were generated covering codons 167, 198, and 200 of the β -tubulin gene of *A. lumbricoides*. Aligned results from the Sanger sequencing demonstrated the wild-type *A. lumbricoides* with 0% SNP frequency. All the three codons targeted (167, 198, and 200) were monomorphic for all the samples (Fig. 2)



Fig. 2. Multiple sequence alignments of β -tubulin amplicon

The first underlined sequence shows the wild-type *A. lumbricoides* (GenBank accession number FJ501301.1) sequences surrounding the target codons 167, 198, and 200 of the β -tubulin gene. Highlighted sequences show 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. A 33% prevalence pre-MDA was observed. This prevalence is higher than a previous 2017 study in the area where the prevalence was 7.3% [13]. However, the sample size of our study was smaller than the 2017 research study. Our study further observed that *A. lumbricoides* was the most prevalent STH, accounting for 94% of the observed prevalence in the study area, followed by hookworms at 3%. However, only 3 mixed infections were observed. Mixed infections can accelerate adverse effects of STHs resulting in malnutrition, anemia, stunted growth and impaired cognitive development [2, 7]. This observation is consistent with a previous study in the region reporting data between 2012 and 2017 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* infections account for the highest morbidity. Of all the positive infections in the study area, none were heavy infections. Majority of the infections (77%) were light infections, suggesting a positive impact of the ten years of deworming in this setting. Studies 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 intensities [25, 26]. Infection intensity directly relates to the morbidity due to STHs [27]. Low worm burdens cause little or no infection amongst the affected individuals. 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, chronic STH infections may dramatically affect physical, mental, and educational development. Although worm burden directly affects children's symptoms, light infections can cause significant symptoms in poor community nutritional status [29, 30]. Heavy-intensity infections by STHs can cause intestinal obstruction, and the nematodes 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 33% to 6%. These samples were collected two weeks post-MDA which provided an optimum timeframe to ensure worm clearance and rule out the possibility of 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 by Mwandawiro and others conducted between 2012 and 2017 observed that the

prevalence started rising again after some time. This could be attributed to environmental and hygienic conditions or infections by parasite strains resistant to the drugs [13]. The persistence of infections despite the mass deworming and the fact that the exercise has been going on for the past ten years may suggest a trend towards resistance associated with SNPs in the β -tubulin gene. Similar to the infections observed pre-MDA, all the infections post-MDA were of light infection intensity (9/12) 75% and moderate infection intensity (3/12) 25%.

This study also sought to detect SNPs in the β -tubulin gene among the positive samples post-MDA. β -tubulin is one of the two protein molecules that join together to make the microtubules. These microtubules are hollow microscopic tubes made up of tubulin dimers (consisting of a β -tubulin and a α -tubulin monomer) and form part of the cytoskeleton [31]. The microtubules play critical roles in the cell including cell division, cell movement, and cell transport[33]. β -tubulin protein molecules act as the target of the BZ, a group of compounds used to treat STHs, and MBZ, the drug used for MDA in this study [34]. These drugs inhibit microtubule polymerization by interfering with cellular structures and functions. This interference leads to the death of the parasites[16]. Mutations in the β -tubulin molecule may 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 eight 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% SNP frequency. No β -tubulin SNPs associated with drug resistance in STH were identified. A recent study conducted in Honduras in 2019 demonstrated similar results[36]. It is noteworthy that even after over ten years of use of benzimidazole drugs in the Kenya school-based deworming program, there is no evidence of drug resistance SNPs. This result supports the continued use of the current anti-helminthic drugs in this setting. However, a previous study Diawara and others in 2013 has, in contrast, identified drug resistant SNPs in the β -tubulin gene of *A. lumbricoides* at codon 167[37]. Different studies on BZA resistance in parasitic nematodes have indicated that this resistance occurs due to SNP in the β -tubulin gene. In these SNPs, amino acid phenylalanine is substituted by tyrosine, which occurs at codons 167 and 200.

Likewise, the amino acid alanine replaces glutamate at codon 198. These mutations have often been associated with drug resistance in STHs [34, 38].

The use of benzimidazoles in the treatment of veterinary nematodes began in the early 1960s. The emergence of BZ-resistance was first reported approximately thirty years later in the 1990s [39][40]. It is encouraging to find that there is no widespread occurrence of drug resistance mutations in human nematodes despite the usage of benzimidazoles for several decades after having first been used in humans in 1974(MBZ) and 1982 (ABZ) [41,42].

5. CONCLUSION

The persistence of some *A. lumbricoides* infections following MDA using mebendazole suggests the drug-resistant phenotype in some nematodes. However, it is also possible that some of the stool samples obtained from the school-aged children could have been from those who did not receive the deworming treatment for one reason or another, including absence from school. It is noteworthy that even after over ten years of use of benzimidazole drugs in the Kenya school-based deworming program, there is no evidence of drug resistance SNPs among *A. lumbricoides* infections. This study supports the continued use of the current anti-helminthic drugs in this setting. We recommend continued use of molecular tools for regular surveillance of drug-resistant parasites as this is essential to ensure the effectiveness of the deworming program.

6. LIMITATIONS

While the findings of this study contribute to the existing literature and offer a foundation for further research, we acknowledge that the study had some limitations. Study samples were collected from only two schools. This limits the generalizability of our findings to the whole of Bungoma County. Additionally, infection comparisons were not conducted pre- and post-MDA on the same children, despite investigation of the same population at both time points. This prevented the calculation of egg reduction rates (ERR), which would have provided valuable information on the efficacy of the medications used.

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, consistent with the previous research 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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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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