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ORIGINAL ARTICLE

The molecular speciation of soil-transmitted helminth eggs collected from school children across six endemic countries

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Background: The diagnosis of soil-transmitted helminths (STHs; *Ascaris*, *Trichuris* and hookworms) is traditionally based on the demonstration of eggs in stool using microscopic techniques. While molecular techniques are more appropriate to speciate STH species they are seldom applied. In this study we speciated STH eggs from stool using molecular techniques to gain insights into the distribution of both human and animal STH species in the human host.

Methods: We speciated 207 STH egg isolates from stool collected during the baseline survey of six drug efficacy trials conducted in Brazil, Cambodia, Cameroon, Ethiopia, Tanzania and Vietnam applying a PCR – restriction fragment length polymorphisms based approach.

Results: DNA of *Ascaris* was detected in 71 (34.3%) samples, of which all were identified as the human roundworm *Ascaris lumbricoides*. In 87 (42.0%) samples, DNA of *Trichuris* spp. was found and further speciation demonstrated the presence of the human *Trichuris trichiura* (100%) and the canine *Trichuris vulpis* (n=7; 8.0%; in Cameroon only). Hookworms were identified in 104 (50.2%) samples, with *Necator americanus* (n=73; 70.2%) being the predominant species followed by *Ancylostoma duodenale* (n=40; 38.5%).

Conclusions: Our study indicates that STH infections in humans are predominantly caused by human STH species. They also suggest that zoonotic transmission occurs on a local scale.

Keywords: *Ascaris lumbricoides*, *Trichiuris trichiura*, *Trichuris vulpis*, *Necator americanus*, *Ancylostoma duodenale*, Zoonosis

Introduction

The soil-transmitted helminths (STHs) are a group of parasitic worms that infect both humans and animals through contact with worm eggs or larvae present in the soil (referring to their common name). The primary species that infect humans are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), *Necator americanus* and *Ancylostoma duodenale* (hookworms).[1] In 2010, it was estimated that approx. 819 million people were infected with *As. lumbricoides*, approx. 465 million with *T. trichiura* and approx. 439 million with hookworms, resulting in a global disease burden of approx. 5.2 million disability adjusted life years (DALYs; 19.9% of the DALYs attributable to neglected tropical diseases).[2] Periodic administration of benzimidazole drugs (albendazole or mebendazole) to at-risk populations has been advocated as a cheap and effective strategy of reducing the worm burden, and hence controlling the related morbidity.[3] Where possible, it is also recommended to use improved water, sanitation and hygiene (WASH) to minimize the rates of re-infection,[4] with the ultimate goal to

eliminate soil-transmitted helminthiasis as a public health problem by 2020.[5]

Although it is commonly accepted that STH infections in humans are caused by one of the four human STH species, studies indicate that a variety of animal STHs can develop to egg-laying adult worms in humans.[6–8] Important animal STH species that are known to cause patent infections in humans, either experimentally or naturally, are *Ascaris suum* and *Trichuris suis* from pigs,[9] and *Ancylostoma ceylanicum* from dogs and cats.[10] In addition, there are a few animal STH species for which human patent infections are only recently confirmed (*Trichuris vulpis* from dogs)[8,11] or suggested (*Ancylostoma caninum*).[12]

The studies designed to speciate STHs derived from humans so far indicate that the role of animals as a zoonotic reservoir should not be underestimated, as animal STH species are attributable for a considerable proportion of the STH infections in humans. In addition, they highlight important geographical variations in the distribution of animal STH species in humans. *Ascaris* infections in developed countries, where human STH species are non-endemic, are almost exclusively caused by pig-to-human transmission (e.g., USA[13]; Denmark[14]; UK[15]; Japan[16]), whereas zoonotic transmission has only been occasionally reported in countries where STHs pose an important burden on public health, such as Uganda[17] (<1% of the human derived *Ascaris* spp. worms were of pig origin), Zanzibar[18] (2%) and China[19] (14%). Zoonotic *Trichuris* spp. infections have been reported in Uganda[20] (10% of the human derived *Trichuris* spp. worms were of pig origin) and Thailand[8] (11% of the speciated *Trichuris* egg isolates from human stool contained *T. vulpis*). *An. ceylanicum* infections are reported in variety of Asian countries, including Cambodia[21] (52% of the speciated hookworm egg isolates derived from human stool contained *An. ceylanicum*), Malaysia[22] (23%), Laos[23] (17%), Thailand[24] (6%) and India[7,25] (5%). Recently, *An. caninum* was also detected in 17% of the egg isolates derived from human stool in India, suggesting that, in contrast to the current knowledge,[26,27] *An. caninum* may be able to develop into egg-laying adult worms in humans.[12] Note that the animal hookworms were at least the second most prevalent hookworm species in each of the aforementioned studies involving hookworms. Albeit based on a small number of hookworm egg isolates, *An. ceylanicum* infections were rather homogenously distributed across Malaysian villages (approx. 20%)[22] whereas it was only found in two of the 50 samples identified positive for hookworm from a tribal area in India (5%).[7] A local scattered distribution was also observed for *An. caninum*, the canine hookworm being identified in seven out of the 10 tribal villages in India (frequency within villages ranging from less than 5% up to 100%).[12]

These studies indeed contribute to a growing body of literature on the role of animals as a reservoir for STH infections in humans. Although the 'One Health' approach has been proposed for *An. ceylanicum*,[10] it remains unclear whether there truly is a need for additional measures to reduce the animal-to-human transmission. Probably the most important reason for the lack of evidence is the use of inadequate tools to speciate STH species in large-scale epidemiological surveys. Traditionally, the diagnosis of STHs is based on the demonstration of eggs in stool.[28] Although the majority of the current microscopic techniques are cheap and easy to use in the field, they do not allow unraveling the importance of animal STH species in humans. This is because, it is impossible to differentiate these animal STH species from their human counterparts on the morphology of the eggs: eggs of both human and animal roundworm (45–75 × 35–50 μm) and hookworms (55–79 × 35–45 μm) are identical.[29,30] Although the eggs of the canine whipworm *T. vulpis* (70–90 × 32–41 μm) are traditionally larger than the human whipworm *T. trichiura* (50–58 × 22–27 μm), speciation of the eggs based on the size remains unreliable. There is an overlap in the length of the eggs of both *Trichuris* species that could mislead diagnosis based on the egg dimension only. Yoshikawa and colleagues reported the presence of both small (57 × 26 μm) and large (78 × 30 μm) eggs in the uteri of adult female *T. trichiura* worms.[31] Moreover, this misdiagnosis may worsen when *T. trichiura* eggs are recovered shortly after treatment, as the egg morphology may change due to the administration of benzimidazoles, increased size being one of the morphological changes.[32] As a consequence of this, reports drawing conclusions on zoonotic *T. vulpis* infections based on the size of eggs should be interpreted with caution.[11,32] With the recent advances in molecular technologies a variety of techniques (e.g., PCR, PCR-restriction fragment length polymorphisms [RFLP] and qPCR[7,8,33,34]) have been developed to molecularly speciate the different STH species, but they are rarely applied. The present study aimed to assess the distribution of both animal and human STH across different geographical settings where STHs are endemic.

Material and methods

Selection of samples

The STH samples used for the present study were collected as part of a multicentric drug efficacy study that was designed to assess the efficacy of a single-oral dose of 500 mg mebendazole against STH infections in children. This study was conducted in six STH-endemic countries across Africa (Cameroon, Ethiopia and Tanzania), Asia (Cambodia and Vietnam) and Latin America (Brazil). The details of this drug efficacy study have been described elsewhere.[35] Each of the different study sites preserved approximately 100 stool samples of subjects excreting eggs of any STH species at baseline (1 g of stool in 10 ml of 70% ethanol). The microscopic detection of eggs in stool was based on the McMaster egg counting method.[36] These samples were subsequently sent to the Laboratory of Parasitology, Ghent University, Belgium for the molecular differentiation of the egg isolates.

Per study site a random set of 40 samples were selected for further molecular speciation, except for Tanzania and Vietnam. Given the high frequency of mixed STH infections, representing *Ascaris*, *Trichuris* and hookworm infections, we only selected 20 samples from Tanzania. For Vietnam, samples were lost during shipment, and as a consequence of this a molecular speciation could only be performed on 27 samples.

Extraction of DNA from eggs in stool

Genomic DNA was extracted from STH eggs using the QIAamp DNA stool mini kit (Hilden, Germany). To this end, 200 μl of the stool suspension (1 g in 10 ml of 70% ethanol) was used to extract DNA according to the manufacturer's recommendations. However, prior to DNA extraction the suspension was subjected

to three freeze-thaw cycles (liquid nitrogen for 2 min and subsequently transferring them to 95°C for 5 min). Every DNA extraction included a negative control sample (water).

Molecular speciation

We applied one general semi-nested PCR separately for each of the three STH genera (*Ascaris*, *Trichuris* and hookworms). The primers for each of these PCRs targeted the ITS-1, 2 and 5.8 s region and were designed using EditSeq and MegAlign (Lasergene, DNASTAR Inc, Madison, WI, USA). All the reactions were performed in a volume of 25 µl containing 2.5 µl DNA, 0.5 µl of each primer (10 mM), 0.5 µl dNTP (10 µM), 1 µl $MgCl_2$ (25 µM), 5 µl GoTaq Flexi buffer, 14.875 µl PCR-grade water and 0.125 µl GoTaq Flexi DNA polymerase. Both a negative (water) and positive (control DNA from corresponding worm species, either worms or eggs: *As. suum*, *As. lumbricoides*, *Trichuris vulpis*, *T. trichiura*, *N. americanus*, *An. duodenale*, *An. ceylanicum* and *An. caninum*) control was included in each run. The following conditions were used: 2 min at 95°C (initial denaturation), 34 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing), 30 s at 72°C (extension), followed by a single step of 10 min at 72°C (final extension). The amplified product was detected using 1.5% agarose gel electrophoresis using ethidium bromide. When no DNA could be detected, the sample was re-analyzed starting with a 1:10 dilution to reduce the impact of inhibitors and improve the detection by PCR. Further speciation was based on RFLP for *Ascaris* and hookworm, and species-specific PCRs for *Trichuris*.

Ascaris

The semi-nested *Ascaris* PCR was performed using the first-round forward primer AsITF-Ext (5′-CCGGGCAAAAGTCGTAACAA-3′) and the second-round forward primer AsITF-Int (5′-TCCGAACGTG CACATAAGTAC-3′) along with the common reverse primer AsITSR (5′-CATATACATCATTATTGTCACGC-3′). These primers were designed using the sequence of *As. lumbricoides* (GenBank accession nos. AB571298, AB571297 and AB571301) and *As. suum* (GenBank accession nos. AB571302 and AB576592). The PCR resulted in a product size of 849 bp. Differentiation between *As. lumbricoides* and *As. suum* was performed as described by Zhu and colleagues.[37] In short, the second-round PCR product was digested using restriction enzyme HaeIII at 37°C for 13 h. HaeIII digests PCR products of *As. lumbricoides* into two (515 bp and 334 bp) and of *As. suum* into three (515 bp, 228 bp and 106 bp). The digested product was detected using 2% agarose gel electrophoresis using ethidium bromide. Figure 1 illustrates the PCR-RFLP analysis for differentiating *As. lumbricoides* from *As. suum*.

Trichuris

The semi-nested PCR was performed using the common forward primer UGTF (5'-TGACAACGGTTAACGGAGAAT-3'), the first-round reverse primer UGTR-Ext (5′-TCAAGTCGCCAAGGACACTC-3′) and the second-round reverse primer UGTR-Int (5′-CGACTCCTGCTT AGGACGAC-3′). These primers were designed using the sequence of *T. trichiura* (GenBank accession nos. GQ301554, GQ301555 and GQ352554), *T. suis* (GenBank accession nos. AM993010, AM993012, AM993014 and AM993016) and *T. vulpis* (GenBank accession no. AM234616). The first-round PCR resulted in a product of 399 bp, while the second-round PCR resulted in a product of 327 bp. Unlike *Ascaris* and hookworm, differentiation of *T. trichiura*/*T. suis* from *T. vulpis* was done using species-specific primers that bind to the interspecies conserved regions of the SSU rRNA region of *Trichuris* genome as described by Areekul and colleagues.[8] This PCR differentiates *T. trichuria*/*T. suis* from *T. vulpis* giving a product size of 207 bp and 212 bp respectively. The amplified product was detected using 1.5% agarose gel electrophoresis using ethidium bromide. Figure 2 illustrates the differentiation of *T. trichiura*/*T. suis* from *T. vulpis* using a nested PCR with species-specific primers. A subset of the *Trichuris* were sequenced and compared with reference sequences using MegAlign (DNASTAR®).

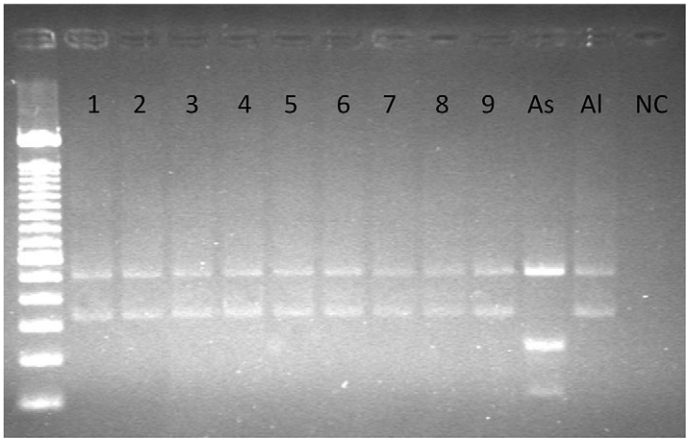

Figure 1. The differentiation of *Ascaris lumbricoides* from *Ascaris suum* applying PCR-RFLP (HaeIII enzyme). Lanes 1 to 9 represent field samples. Al: positive control for *As. lumbricoides* (515 bp and 334 bp); As: positive control sample for *As. suum* (515 bp, 228 bp and 106 bp); NC: negative control.

Hookworm

A semi-nested hookworm PCR was performed as previously described by George and colleagues.[7] The first round of this PCR resulted in an amplicon of 597 bp and 449 bp for *N. americanus* and *Ancylostoma* spp, respectively. While the second PCR product resulted in an amplicon of 552 bp for *N. americanus* and 404–408 bp for *Ancylostoma* spp. Further characterization of *Ancylostoma* spp. was done using RFLP. To this end, the second-round PCR products were digested using the restriction enzymes MvaI and Psp1406I at 37°C for 13 h. MvaI digests PCR products of *An. ceylanicum* into two (340 bp and 64 bp), but does not digest *An. duodenale* and *An. caninum*. Psp1406I digests *An. duodenale* PCR products into two (255 bp and 149 bp), but does not digest *An. ceylanicum* and *An. caninum*. The digested products were detected using 2% agarose gel electrophoresis using ethidium bromide.

Ethical approval

The parents of all subjects included in the studies signed an informed consent form. In Brazil and Ethiopia an informed consent form was obtained from children aged 10 or 11 years and

above. In Cambodia and Ethiopia, a verbal assent was obtained from all children and these procedures were approved by their respective Institutional Review Boards. This trial was registered under the ClinicalTrials.gov identifier no. NCT01379326.

Results

In the present study a total of 207 STH isolates from an equal number of subjects were examined for the presence of both human and animal STH species. DNA was detected for at least one of the three general semi-nested PCRs in 165 (79.7%) samples. DNA of *Ascaris* was detected in 71 (34.2%) samples, of which all were identified to be the human *As. lumbricoides*. In 87 (42.0%) samples, DNA of *Trichuris* spp. was found and further determination of the species using species-specific primers (and sequencing a subset of the samples) indicated the presence of *T. trichiura* in all the samples. In addition, DNA of the canine *T. vulpis* was detected in seven samples from Cameroon. Hookworm DNA was detected in 104 samples (50.2%). The majority of hookworm isolates were identified as *N. americanus* (n=73; 70.2%) followed by *An. duodenale* (n=40; 38.5%). No animal hookworm species were found. Mixed *N. americanus* and *An. duodenale* were observed in nine samples (8.7%). The distribution of the different STH species is provided in Table 1.

Table 2 reports the distribution of mono and mixed STH infections, highlighting that in the majority of the samples, DNA of at least two different STH species was found (86/165). Infections with two STH species were found in 64 samples, triple STH infections in 17 samples and quadruple STH infections in five samples. The most common double mixed infections were that of *As. lumbricoides* and *T. trichiura*, the most common triple infection was a combination of *As. lumbricoides*, *T. trichiura* and *N. americanus*.

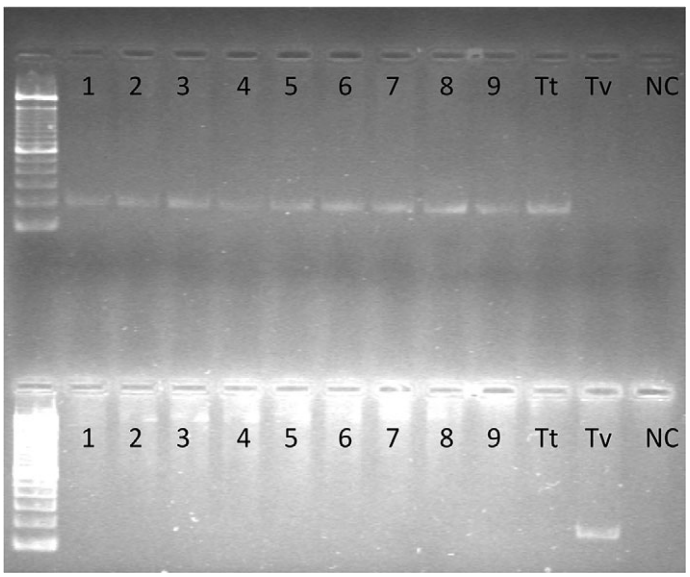

Figure 2. The differentiation of *Trichuris* spp. using a nested PCR based on species-specific primers. Top gel: the *T. trichiura/T. suis* specific nested PCR; Bottom gel: the *T. vulpis* specific PCR. Lanes 1 to 9 represent field samples. NC: negative control; Tt: positive control for *T. trichiura*; Tv: positive control for *T. vulpis*.

Discussion

It is traditionally accepted that STH infections in humans are caused by the human STH species only (*As. lumbricoides*, *T. trichiura*, *N. americanus* and *An. duodenale*). However, recent epidemiological studies applying molecular techniques indicate that the role of animals as a zoonotic reservoir for STH infections in humans should not be underestimated.[8–10,12] In the present study we molecularly speciated STH isolates collected from children during a drug efficacy study in six STH-endemic countries across Africa, Asia and Latin America, with the aim to gain insights into the distribution of both human and animal STH species.

Our results highlighted that the STH infections were almost exclusively caused by human STH species. Only in Cameroon, DNA of the canine *T. vulpis* was detected in 7 out of 23 subjects infected with *Trichuris* (Table 1). Our findings are in line with molecular studies conducted in northwestern Thailand where they found six out of 56 subjects excreting eggs of *T. vulpis*.[8] These findings contribute to the evidence that *T. vulpis* may cause patent infections in humans. However, the burden of disease caused by this animal STH species in humans is unclear. In the present study, no animal roundworms and hookworms were identified. The absence of zoonotic *Ascaris* transmission confirms the earlier findings in the literature, indicating that

Table 1 The distribution of *Ascaris*, *Trichuris* and hookworm spp. in six endemic countries

Country	n	*Ascaris*		*Trichuris*		Hookworm	
		A. lumbricoides	*A. suum*	*T. trichiura*	*T. vulpis*	*Necator americanus*	*Ancylostoma duodenale*
Brazil	40	20	0	19	0	17	3
Cambodia	40	0	0	0	0	21	10
Cameroon	40	17	0	23	7	3	14
Ethiopia	40	15	0	15	0	10	3
Tanzania	20	14	0	20	0	8	10
Vietnam	27	5	0	10	0	14	0
Total	207	71	0	87	7	73	40

Table 2. The distribution of mixed infections with *Ascaris*, *Trichuris* and hookworm spp. in six endemic countries

Species	Overall	Brazil	Cambodia	Cameroon	Ethiopia	Tanzania	Vietnam
Mono infections (n=79)							
As. lumbricoides	16	6	0	2	6	0	2
T. trichiura	19	4	0	7	7	1	0
T. vulpis	0	0	0	0	0	0	0
An. duodenale	10	0	4	4	2	0	0
N. americanus	34	8	15	0	6	0	5
Double infections (n=64)							
As. lumbricoides+T. trichiura	22	8	0	4	6	3	1
As. lumbricoides+N. americanus	7	4	0	1	2	0	0
As. lumbricoides+An. duodenale	5	0	0	4	1	0	0
T. trichiura+T. vulpis	3	0	0	3	0	0	0
T. trichiura+N. americanus	13	2	0	0	2	2	7
T. trichuira+An. duodenale	8	2	0	3	0	3	0
N.americanus+An. duodenale	6	0	6	0	0	0	0
Triple infections (n=17)							
As. lumbricoides+T. trichiura+T. vulpis	1	0	0	1	0	0	0
As. lumbrcoides+T. trichiura+N. americanus	9	2	0	1	0	4	2
As. lumbricoides+T. trichiura+An. duodenale	6	0	0	1	0	5	0
T. trichiura+N. americanus+An. Duodenale	1	1	0	0	0	0	0
Quadruple infections (n=5)							
As. lumbricoides+T. trichiura+N. americanus+An. duodenale	2	0	0	0	0	2	0
As. lumbricoides+T. trichiura+T. vulpis+N. americanus	1	0	0	1	0	0	0
As. lumbricoides+T. trichiura+T. vulpis+An. duodenale	2	0	0	2	0	0	0

An.: Ancylostoma; As.: Ascaris; N.: Necator; T.: Trichuris.

pig-to-human transmission is mainly found in countries where human STH are not endemic (see also Introduction). Moreover, in some countries pigs are rare or absent due to cultural habits (e.g., Ethiopia and Tanzania [Pemba]), and hence zoonotic transmission is unexpected, though pig-to-human transmission cannot be entirely excluded.[18] This is in contrast for animal hookworm species, were evidence of zoonotic transmission was absent, particularly for *An. ceylanicum* in Asian countries. This animal hookworm has been previously detected in humans from Asian countries, such as Cambodia (52% of the hookworm egg isolates derived from human stool),[21] highlighting once more the geographical variation in the transmission of zoonotic hookworm species.[7,12,22,38] At this stage it is difficult to explain this geographical variation. The most apparent factors might be differences in the prevalence of animal STHs in their natural hosts; the population size of dogs or cats; and the way these animals and human populations interact with each other. The latter is probably the most important factor, as animals are often both abundant and infected in STH-endemic countries. For example, in Vietnam *An. ceylanicum* are highly prevalent in dogs (half of the dogs are infected with hookworms, of which more than 60% identified as *An. ceylanicum*).[34]

The present study has three major limitations. First, this study was embedded into a multicentric clinical trials designed to assess the efficacy of mebendazole against STH infections in children. As a consequence of this, our STH egg isolates do not represent a random sample from the total population of STHs. Second, we deployed general genus primers into our PCR protocols for *Ascaris*, *Trichuris* and hookworms. An important disadvantage of this approach is that it will amplify DNA of the most abundant species, and hence we might have missed mixed infections with human and animal STH species (e.g., *An. ceylanicum*). Third, we are not able to draw conclusions on the absence of *T. suis* infections, as the applied primer set did not allow to differentiate the swine from the human whipworm. However, sequence results of a selection of *T. trichiura/T. suis* products only revealed the presence of *T. trichiura*. Finally, we were not able to quantify the STH species infections within a sample; rather we confirmed the presence or absence of different STH species. This quantification of STHs would be particularly interesting to assess the relative contribution of *T. vulpis* and *T. trichiura* in the isolates from Cameroon.

Conclusions

In conclusion, our study indicates that STH infections in humans are predominantly caused by human STH species, and suggests that zoonotic transmission mainly occurs on a more local scale. As a consequence of this, there is no need to adapt the current recommended deworming programmes, rather it will be important to further explore the speciation of both human and animal

derived STHs to identify hot spots of zoonotic transmission, and to subsequently develop and implement local control strategies to reduce animal-to-human transmission.

Authors' contributions: SG, PG, GK, and BL designed the molecular speciation; MA, DE, AM, JV and BL designed the collection of isolates; SMA, JMB, ZM, SH, L-A T-T, NTH, JV and BL collected the isolates; SG, PG, GK and BL analyzed and interpreted the data; SG and BL wrote the manuscript; PG and GK reviewed the manuscript. All authors read and approved the final manuscript. BL is the guarantor of the paper.

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Competing interests: None declared.

Ethical approval: The overall protocol of the mebendazole trial was approved by the Ethic committee of the Faculty of Medicine, Ghent University (reference no. 2011/374), which was followed by a local ethical approval at each trial site. For Brazil, ethical approval was obtained from the Institutional Review Board (IRB) from Centro de Pesquisas René Rachou (reference no. 21/2008). For Cambodia, from the National Ethic Committee for Health Research (reference no. 185). For Cameroon, from the National Ethics Committee (reference no. 147/CNE/DNM/11). For Ethiopia, from the IRB of Jimma University (reference no. RPGE/09/2011). For United Republic of Tanzania, from the Zanzibar Health Research Council (reference no. 20/ZAMREC/0003/JUNE/2012), and for Vietnam, by the Ethical Committee of National Institute of Malariology, Parasitology and Entomology and the Ministry of Health (reference no. 752/QD-VSR).

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