Simultaneous Detection and Quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in Fecal Samples Using Multiplex Real-Time PCR

Jaco J. Verweij, Eric A. T. Brienen, Juventus Ziem, Lawrence Yelifari, Anton M. Polderman, and Lisette Van Lieshout

Leiden University Medical Center, Department of Parasitology, Leiden, The Netherlands; Ministry of Health, Upper East Region, Bolgatanga, Ghana; University for Development Studies, School of Medicine and Health Sciences, Tamale, Ghana

Abstract. A multiplex real-time PCR was developed and evaluated for the simultaneous detection of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in fecal samples. Using well-defined control samples (N = 150), known positive fecal samples (N = 50), and fecal samples from an area in Ghana where human infections with all 3 nematode species are endemic (N = 339), the method proved to be highly specific and sensitive. Cycle threshold (Ct) values, reflecting parasite-specific DNA load, showed significant correlation with the intensity of infection as measured by microscopy using Kato–Katz fecal smears or by species specific third-stage larval count after coproculture. The multiplex real-time PCR described combined with the simple fecal sample collection procedure and the potential for high throughput makes this approach a powerful diagnostic tool to study species-specific transmission patterns of human hookworm-like infections. Moreover, this procedure facilitates monitoring of intervention programs and allows species-specific detection of treatment failure following rounds of mass treatment.

INTRODUCTION

Ancylostoma duodenale and Necator americanus are soil-transmitted helminths responsible for the estimated 740 million human hookworm infections worldwide. N. americanus is considered the more common infection, whereas A. duodenale infection appears to be more geographically restricted. Hookworm infections are a leading cause of anemia, and its severity is directly correlated with the number of adult hookworms in the human intestine. Although severity of anemia has also been shown to correlate with the hookworm species involved. Accurate data on the precise distribution of the two species are lacking because species differentiation is usually not performed. Mass treatment using benzimidazole anthelmintics results in improvement of hookworm-related morbidity rates in children living in endemic areas; however, concerns exist regarding the sustainability of such programs and the emergence of resistance.

Human oesophagostomiasis, caused by the strongylid nematode Oesophagostomum bifurcum, has long been considered a rare zoonosis. However, in northern Togo and Ghana, prevalence of O. bifurcum infections can reach 50% or more in some villages and is strongly associated with hookworm infections, with co-infection rates approaching 100%. O. bifurcum-induced nodular pathology has been demonstrated in up to 50% of the infected individuals and 2% of these may eventually require surgical intervention if untreated. In a recent study, prevalence of O. bifurcum infection and severity of Oesophagostomum-induced nodular pathology were reduced significantly after 2 rounds of mass treatment with albendazole. However, it is unclear whether this disease can really be controlled in this way, as many details of the transmission patterns and the risk factors involved are still unknown for this parasite. There is clearly a need for sensitive and specific diagnostic procedures to detect each of these 3 nematodes, to measure the intensity of infections, to study transmission patterns of each species, and to monitor the effects of intervention programs and possible treatment failures.

The most common method used to describe hookworm infections within a population involves counting the number of eggs per gram of feces by the Kato–Katz thick-smear technique. Microscopic examination of Kato–Katz slides for the presence of hookworm-like eggs is hampered by the fact that the slides must be read within 30 minutes or many eggs will no longer be visible. Moreover, species identification in hookworm and O. bifurcum infections is not possible due to the fact that the eggs of all 3 species are morphologically indistinguishable. Therefore, coproculture is required to allow eggs to develop and hatch, producing the characteristic third-stage larvae (L3s) needed for differential diagnosis. Coproculture-based diagnostics are completely dependent on the availability of fresh stool specimens, and differentiation of the L3s, especially those of A. duodenale and N. americanus, requires a highly skilled microscopist. Large-scale application of coproculture in epidemiologic surveys is also hampered by the fact that these cultures have to be kept in closed Petri dishes for 1 week. At tropical temperatures, this frequently leads to the development of maggots and overgrowth of fungi, and coprocultures must be performed in duplicate to obtain more reliable results.

PCR-based methods for specific detection of the nematodes in fecal samples have been used successfully for all 3 species. In these methods, a nested PCR approach consisting of 2 consecutive rounds of amplification was needed to achieve adequate sensitivity. Furthermore, the second (species-specific) PCR had to be performed for each species separately, and PCR products had to be visualized on ethidium bromide–stained agarose gels. As a consequence, amplification and detection of DNA were prone to contamination as well as time-consuming and expensive. Moreover, the endpoint reading on agarose gels yielded no quantitative information. Introduction of real-time PCR using fluorescent detection probes reduces the risk of contamination and substantially reduces labor time and reagent costs, through the possibility of combining assays for the detection of different species.
targets into 1 reaction. Real-time PCR also offers the possibility of quantification using the threshold cycle; this is the amplification cycle in which the level of fluorescent signal exceeds background fluorescence, and this correlates with the initial copy number at the beginning of the reaction.

We have developed a multiplex real-time PCR for the simultaneous detection, differentiation, and quantification of *A. duodenale*, *N. americanus*, and *O. bifurcum* infection in fecal samples. Additionally, an internal control for detection of amplification inhibition by fecal contaminants was included in the assay. The performance of the assay was evaluated using a range of well-defined fecal samples and controls. Samples collected in an area known to be endemic for all 3 species were used to correlate Ct values from the species-specific PCRs with the quantitative results of Kato–Katz smear analysis and larval counts resulting from coproculture.

**MATERIALS AND METHODS**

**Controls.** To establish the PCR assays, genomic DNA was isolated using the QIAamp Tissue Kit (QIAGen, Hilden, Germany) from individual adult worms of *N. americanus* and *O. bifurcum* and L3s of *A. duodenale*. Worms were obtained from feces of infected patients after treatment with praziquantel, as described previously.7 Specificity of the PCR was evaluated using DNA isolated from adult *Ascaris lumbricoides*, *Trichuris trichiura*, and Schistosoma mansoni worms as template. In addition, *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, Cryptosporidium parvum, Cyclospora cayetanensis, Enterocytozoon bieneusi, and Encephalitozoon intestinalis DNAs20 were also tested, as were DNAs obtained from 17 different bacterial and yeast cultures: *Bacillus cereus*, *Campylobacter jejuni*, *Campylobacter upsiliensis*, Candida albicans, Escherichia coli O157, Enterobacter aerogenes, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella typhimurium, Shigella boydi, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Staphylococcus aureus, and *Yersinia enterocolitica*.

Eighty stool DNA samples from different patients were tested in which *E. histolytica* (N = 20), *E. dispar* (N = 20), *G. lamblia* (N = 20), or *C. parvum/C. hominis* (N = 20) were detected by microscopy and confirmed by species-specific PCR.21,22 Next, 40 unprocessed stool samples were tested from patients with negative microscopy using formalin–ether sedimentation and modified acid-fast staining and a negative Giardia-antigen test. In these negative samples, 2 subsequent stool samples from these patients also tested negative by all conventional methods.

**Field samples.** For a preliminary evaluation of the PCR, DNA isolated from 50 human fecal samples was used, for which coproculture had shown L3s of *N. americanus*, *O. bifurcum*, and/or *A. duodenale*. These samples originated from three different geographical regions. From northern Togo, known to be endemic for both *N. americanus* and *O. bifurcum*,15 samples with L3s of both species and 15 samples with *N. americanus* L3s only were used. From central Ghana, 10 samples with *N. americanus* L3s were used; in this region, *N. americanus* is endemic, and human infections with *O. bifurcum* are unknown.19 From Malawi, 10 samples were used in which *A. duodenale* L3s were identified after coproculture.

For subsequent evaluation, DNA isolated from stool samples (N = 339) collected in a number of communities situated in northern Ghana was used to evaluate the sensitivity of the real-time PCR as compared with direct smear, Kato–Katz, and coproculture. The association between the quantitative outcome of real-time PCR represented as threshold values and the intensity of infection as measured by classic microscopy using Kato–Katz was also determined. In northern Ghana, *N. americanus*, *O. bifurcum*, and *A. duodenale* are all present, with *N. americanus* the predominant species. However, prevalence and intensity of infection of these 3 nematodes differ substantially at the community level.10,17,23 Samples were selected for DNA isolation and PCR analysis based on the availability of results for a duplicate coproculture, in combination with either a Kato–Katz examination (N = 281) or direct smear (N = 58). Ethical considerations and the method of sample collection are described in detail elsewhere.10,23

**Microscopy.** Microscopic examination of the field samples for the presence of hookworm-like ova was performed either by direct smear or by counting the number of eggs in a single 25-mg Kato–Katz smear.14 Coproculture, differentiation of *O. bifurcum* and hookworm L3s, and quantification were carried out as previously described.27,28 Fecal samples were transported to Leiden for DNA isolation either frozen or suspended in ethanol and stored at room temperature.

**DNA isolation.** For DNA isolation, 200 μL of fecal suspension (+ 0.25 g/mL ethanol) was centrifuged, and the pellet was washed twice with 1 mL of PBS. After centrifugation, the pellet was resuspended into 200 μL of 2% polyvinylpyrrolidone (PVPP, Sigma, St. Louis, MO) suspension and heated for 10 minutes at 100°C. After sodium dodecyl sulfate–proteinase K treatment (2 hours at 55°C), DNA was isolated with the QIAamp Tissue Kit spin columns (QIAGen).18 In each sample, 105 PFU/mL phocin herpes virus type 1 (PhHV-1) was added within the isolation lysis buffer to serve as an internal control.24

**PCR amplification and detection.** PCR primers and minor groove binding (MGB) detection probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and the Internal Transcribed Spacer 2 (ITS2) sequence of each species. The *N. americanus*-specific primers, Na58F and NA158R (Biolegio, Malden, The Netherlands), amplify a 105-bp fragment of the ITS2 sequence, and the MGB-Taqman probe Na81MGB (Applied Biosystems, Warrington, U.K.) was used to detect *N. americanus*-specific product. The *A. duodenale*-specific primers Ad125F and Ad195R (Biolegio) amplify a 71-bp fragment of the ITS2 sequence, and the MGB-Taqman probe Ad155MGB (Applied Biosystems) detects the *A. duodenale*-specific product. The *O. bifurcum*-specific primers Ob31F and Ob136R (Biolegio) amplify a 105-bp fragment of the ITS2 sequence, and the MGB-Taqman probe Ob63MGB (Applied Biosystems) detects the *O. bifurcum*-specific product.

The PhHV-1–specific primers and probe24 set consisted of forward primer PhHV-267s, reverse primer PhHV-337as, and the double-labeled probe PhHV-305tq (Biolegio).

Serial 10-fold dilutions of DNA extracted from each pathogen were tested with and without the presence of internal control DNA to estimate the latter’s influence. Each dilution series was also tested with and without the other target DNAs to assess the assay’s ability to detect mixed infections.
Amplification reactions were performed in a volume of 25 μL with PCR buffer (HotstarTaq master mix, QIagen), 5 mM MgCl₂, 2.5 μg of bovine serum albumin (Roche Diagnostics Nederland B.V., Almere, The Netherlands), 1.5 pmol of each A. duodenale-specific primer, 1.5 pmol of each O. bifurcum-specific primer, 5 pmol of each N. americanus-specific primer, 3.75 pmol of each PhHV-1-specific primer, 2.5 pmol of A. duodenale-specific MGB-Taqman probe, 2.5 pmol of O. bifurcum-specific MGB-Taqman probe, 2.5 pmol of N. americanus-specific MGB-Taqman probe, 2.5 pmol of PhHV-1-specific double-labeled probe, and 5 μL of the DNA sample. Amplification consisted of 15 min at 95°C followed by 50 cycles of 15 s at 95°C, 60 s at 60°C. Negative and positive control samples were included in each amplification run.

Amplification, detection, and data analysis were performed with the AB7500 real-time PC system (Applied Biosystems). All primers and detection probes are described in Table 1.

**Data analysis.** For statistical analyses, data were entered in SPSS 11.01 (SPSS Inc., Chicago, IL). Continuous variables were described by range and median of all positive cases and compared between groups by the Mann-Whitney rank-sum test. Spearman’s rank correlation (ρ) was used for calculation of the concordance between Ct values, reflecting parasite specific DNA loads, and intensity of infection as determined by microscopy (i.e., hookworm-like eggs per gram of feces or species-specific L3 counts). For this purpose, “zero values” in Ct values, eggs per gram (epg), and L3 counts were redefined as 45, 20, and 0.5, respectively. Statistical significance was set at P < 0.05.

## RESULTS

The real-time PCRs were optimized first as monoplex assays with 10-fold dilution series of A. duodenale, N. americanus, and O. bifurcum DNA, respectively. The monoplex real-time PCRs were thereafter compared with the multiplex PCR with the PhHV internal control. The cycle threshold (Ct) values obtained from testing the dilution series of each pathogen in both the individual assay and the multiplex assay were similar, and the same analytical sensitivity was achieved. The individual performance of the assays was not influenced by the presence of DNA from the internal control, or by the presence of DNA from the other targets.

The specificity of the real-time multiplex PCR was evaluated using a range of parasite and bacterial control DNAs (N = 30), 80 DNA extracts derived from feces positive for E. histolytica, E. dispar, G. lamblia, or C. parvum/C. hominis, and 40 DNA extracts derived from feces of individuals with no known history of parasitic infections. No amplification of A. duodenale, N. americanus, or O. bifurcum DNA was detected in any of these 150 samples, only the amplification of the internal control was detected at the expected Ct value of ≈ 30.

Performance of the multiplex real-time PCR was first evaluated using DNA isolated from 50 stool samples. N. americanus-specific amplification was detected in all 40 DNAs isolated from feces shown to contain N. americanus L3s after coproculture. O. bifurcum-specific amplification was demonstrated in 13 of 15 samples in which O. bifurcum L3s were found. A. duodenale-specific amplification was detected in all 10 samples with A. duodenale L3s. In addition, O. bifurcum-specific amplification was detected in 7 of 15 DNA samples known to contain N. americanus L3s and in which no O. bifurcum L3s were found. These fecal samples were all from northern Togo, where human oesophagostomiasis is an endemic disease. In contrast, in 10 fecal samples from central Ghana, where human infection with O. bifurcum is unknown, no O. bifurcum-specific amplification was demonstrated.

Samples (N = 339) from northern Ghana were used for a more detailed evaluation of the real-time multiplex PCR. Hookworm-like eggs were demonstrated by direct smear in 20 of 58 (34.5%) samples or by Kato–Katz examination in 211 of 281 samples (75.1%). The egg counts ranged from 40 to 29,480 epg (median 720). After coproculture, L3s of hookworm were seen in 268 samples (79.1%, range 1–1,601, median 63) and L3s of O. bifurcum in 83 samples (24.5%, range 1–575, median 11). In total, 271 samples (79.9%) showed L3s of at least 1 of the nematodes.

In this study group, specific amplification of A. duodenale was detected in 23 samples (6.8%), N. americanus in 300 samples (88.5%), and O. bifurcum in 103 samples (30.4%). Specific amplification of A. duodenale and N. americanus to-

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**Table 1**

<table>
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<tr>
<th>Target organism</th>
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<th>Oligonucleotide sequence</th>
<th>GenBank accession no. or literature reference of target sequence</th>
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<td>N. americanus</td>
<td>Na558f</td>
<td>5'-CTGTTGTGTCGACGTCATGTGC-3'</td>
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gether was detected in 21 samples, and amplification of both *N. americanus* and *O. bifurcum* in 101 samples. Specific amplification of all 3 species in the same sample was not found. Further analysis of the *A. duodenale* PCR was not possible due to the low number of infected cases in the study group.

Table 2 summarizes the comparison between microscopy and the multiplex real-time PCR. *A. duodenale, N. americanus,* and/or *O. bifurcum*-specific amplification was detected in all 20 samples in which hookworm-like eggs were seen in the direct smear. In addition, specific amplification occurred in 26 of 38 samples in which no hookworm-like eggs were seen with microscopy. Specific amplification was also detected in 210 of 211 samples in which hookworm-like eggs were seen in the Kato–Katz smear, as well as in 48 of 70 samples in which no hookworm-like eggs were seen. Specific amplification was detected in 267 of 271 samples known to contain L3s of one or more species and in 37 of 68 samples in which L3s were not found after coproculture.

In 263 of 268 fecal samples with hookworm L3s, *N. americanus*-specific amplification was detected with Ct values between 20.3 and 38.9 (median 27.6); 37 of 71 fecal samples in which no hookworm L3s were found in coproculture showed *N. americanus*-specific amplification (Ct median 34.2, range 26.3–41.3). *O. bifurcum*-specific amplification was demonstrated (Ct median 28.7, range 22.5–41.3) in 71 of 83 fecal samples known to contain *O. bifurcum* L3s and in 32 of 256 DNA samples from stools in which no *O. bifurcum* L3s were found in coproculture (Ct median 35.0, range 28.3–41.2). Ct values for *N. americanus* and *O. bifurcum* PCRs were significantly lower in those samples in which the corresponding L3s were found compared with coproculture negatives (*P* < 0.001).

Ct values from the *N. americanus* PCR showed a significant correlation (*p* = −0.76; *P* < 0.001) with the quantitative interpretation of the Kato–Katz smear (epg) in 148 fecal samples in which mixed infection with *O. bifurcum* or *A. duodenale* was excluded based on the results of PCR and/or culture (Figure 1A). There was also a highly significant correlation between the Ct values as detected in the *N. americanus* PCR in fecal samples with the quantitative interpretation of species-specific larval count in coproculture (N = 171; *p* = −0.69; *P* < 0.001) (Figure 1B).

<table>
<thead>
<tr>
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<th><em>A. duodenale, N. americanus,</em> and <em>O. bifurcum</em> multiplex PCR</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td><strong>Smear</strong></td>
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<td>46 (79.3)</td>
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<td><strong>Coproculture</strong></td>
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<tr>
<td>Positive</td>
<td>267 (98.5)</td>
</tr>
<tr>
<td>Negative</td>
<td>37 (54.4)</td>
</tr>
</tbody>
</table>

* One hookworm-like egg seen in Kato–Katz.
† 1–5 third-stage larvae found in 1 of 2 coprocultures.

The coefficient of correlation between Ct value of *O. bifurcum* PCR and *O. bifurcum* larval count was 0.68 (N = 113, *P* < 0.001). Further statistical analysis of the *O. bifurcum* PCR in single infections was not possible, as this species is almost always found together with *N. americanus*. Also, in this group of mixed infections, the *N. americanus* PCR showed good correlation with the larval count in coproculture (*p* = −0.71; *P* < 0.001). Mixed infections of *A. duodenale* and *O. bifurcum* were not detected in this study.
DISCUSSION

Hookworm infection is one of the most common chronic infections affecting millions in the tropics and subtropics, mostly in areas of poverty. Anemia and malnutrition as a consequence of these infections are directly correlated with the intensity of infection and the specific species involved. Although mass treatment with anthelmintics has been proven to reduce morbidity caused by hookworm infection, questions have been raised concerning the sustainability of long-time repeated treatments and the emergence of resistance. The long-term effects of mass treatment on the rate of infection and morbidity can be expected to differ among the species involved. The most commonly used tool to measure intensity of infection, egg count with the Kato–Katz method, does not allow differentiation of _A. duodenale_ and _N. americanus_. To achieve this, labor-intensive coproculture and microscopic differentiation of third-stage larvae (L3s) techniques must be performed. In an area in which 3 nematode species can cause human infection and pathology, the multiplex real-time PCR for the detection of _A. duodenale_, _N. americanus_, and _O. bifurcum_ presented in this study has proven to be a specific and sensitive alternative tool for detection and measurement of infection intensity for the 3 species.

Using well-defined DNA and stool samples as controls, and 50 fecal samples known to contain L3s of at least 1 of the 3 species after coproculture, the multiplex real-time assay achieved 100% specificity and sensitivities of 100%, 100%, and 86.7% for the detection of _N. americanus_, _A. duodenale_, and _O. bifurcum_, respectively. Moreover, _O. bifurcum_ DNA was amplified in 7 of 15 samples from an area known to be endemic for human _O. bifurcum_ infections in which coproculture did not reveal this species’ L3s.

Inhibition of PCR by fecal constituents is known to be a serious problem. In all control and study stool samples tested, amplification of the internal control was detected. Hence, there was no evidence of inhibition of amplification in any of these samples using this DNA isolation method.

In a further evaluation using 339 fecal samples from a region where all 3 nematodes are known to be transmitted, the multiplex real-time PCR for the specific detection of _A. duodenale_, _N. americanus_, and _O. bifurcum_ DNA again showed excellent sensitivities of 100%, 99.5%, and 98.5%, as compared with direct microscopy, Kato–Katz smear examination, and the outcome of coproculture, respectively (Table 2). Additionally, _N. americanus_ and _O. bifurcum_ DNA was amplified in samples in which eggs were missed by microscopy and in samples for which coproculture did not reveal larvae. Knowing the high variability in egg excretion and the outcome of coproculture, these are not very surprising findings. The excretion and distribution of parasitic DNA in feces is expected to be less variable than the number of eggs. This has already been shown for the amplification of protozoan-specific DNA when compared with the microscopic detection cysts or oocysts from _G. lamblia_ and _Cryptosporidium_. Further studies to compare the outcome of hookworm egg count performed with Kato–Katz microscopy and real-time PCR in 3 consecutive samples from the same subject are being planned for the near future. It is possible that those cases in which only _O. bifurcum_ DNA was detected harbored tissue-dwelling larval stages causing disease but not excreting eggs. Indeed, negative coprocultures are frequently seen in individual patients with Dapaong tumors. The false-negative real-time PCR results were not caused by inhibition of the amplification by fecal components. One possible explanation could be that the amount of stool used in coproculture is 30 times greater than that used for DNA isolation. Also, the possibility of misidentification of a single egg or larva by microscopy cannot be excluded.

A highly significant correlation was demonstrated between the Ct values of the _N. americanus_ PCR and both egg counts and the number of _N. americanus_ L3s found in coproculture, with or without exclusion of mixed infections. Similarly, correlation between _O. bifurcum_ L3s and the Ct values of the _O. bifurcum_-specific PCR proved to be excellent and highly significant.

Mixing of stool samples with ethanol allows them to be transported and stored at room temperature, and the increasing number of research centers with facilities for real-time PCR, including some in hookworm-endemic countries, makes it possible to use the procedure as described in this paper to monitor the species-specific effects of intervention programs. Obviously, this procedure will not be appropriate as a routine diagnostic tool in clinical settings in these countries.

In northern Ghana, where human infections with 3 nematode species—_A. duodenale_, _N. americanus_, and _O. bifurcum_—are endemic, simultaneous detection of species-specific DNA in fecal samples using multiplex real-time PCR proved to be highly specific and sensitive. This real-time PCR assay also offers species-specific quantification as a reflection of intensity of infection, as is normally obtained from egg or larval counts.

Received December 21, 2006. Accepted for publication June 3, 2007.

Acknowledgments: Collection of data and material would not have been possible without the tremendous efforts of field team workers Bayita Albano, Daniel Laar, Leonard Yelifari, Mohammed Aswel, Moses Kolan, and Mathilda Abugri and Dutch students Welmoed Silvis, Bram Diederik, Gabrielle van Ramshorst, Esther Hamoen, and Rutger van der Meer. In addition, we are most grateful to Robert ten Hove and Maarten Eldering for laboratory assistance. The Severe Anaemia Project team of the Malawi–Liverpool–Wellcome Trust Clinical Research Program is kindly acknowledged for providing _A. duodenale_-positive fecal samples. The authors thank Gilbert Dery and Dr. Von Asigri (Parasitic Diseases Research Center, Tamale, Ghana) for logistical assistance and Coby Blokamp for training and advising the field team. The Ghana Health Service in Bolgatanga and Tamale are gratefully acknowledged for their support. The authors also thank Dr. C. Graham Clark from the London School of Hygiene and Tropical Medicine for his advice and comments in the final version of the manuscript.

Financial support: Financial support was provided by the Dutch Foundation for the Advancement of Tropical Research (WOTRO-NWO) and the _Oesophagostomum_ Research Intervention Project in Northern Ghana.

Authors’ addresses: Jaco J. Verweij, Eric A. T. Brienen, Anton M. Polderman, and Lisette Van Lieshout, Leiden University Medical Center, Department of Parasitology, Leiden, The Netherlands, Telephone: +31-71-5265080, E-mail: j.j.verweij@lumc.nl. Juvenus Ziem, University for Development Studies, School of Medicine and Health Sciences, Tamale, Ghana, Telephone: +233-24-4753335. Lawrence Yelifari, Ministry of Health, Upper East Region, Bolgatanga, Ghana.

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