Tracking helminths : from molecular diagnostics to mechanisms behind immune polarization

Kaisar, M.M.

Chapter 2

Multiplex real-time PCR as opposed to stool microscopy for studying and comparing the distribution of Soil-transmitted helminths within and between population living in endemic regions

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Submitted

Backgroud cover: Helminths worms and eggs. Credit to Eric Brienen

ABSTRACT

Monitoring soil-transmitted helminth (STH) infections is usually done via stool microscopy. Although relatively simple, this approach is highly observer- and laboratory procedure-dependent. Here we evaluated data from two community-based surveys (Indonesia, Mozambique). Stool microscopy was compared with real-time PCR data. Subsequently, PCR findings were used to analyse potential risk-factors for STH infections in the Indonesian study population. In Indonesia a formol-ether concentration procedure was applied some months after stool collection at Flores-Island (N=1087), whereas in Beira (Mozambique) five different microscopy methods were used on the day of collection (N=303). Aliquots were transferred to a centralized laboratory for the detection and quantification of parasite-specific DNA of *Ancylostoma* spp., *Necator americanus, Ascaris lumbricoides* and *Strongyloides stercoralis*. For the Indonesian study population socio-demographic data and household properties were compared with parasitological outcomes. In the Indonesian samples, real-time PCR detected considerably more positive cases than microscopy, in particular for the hookworm species (73% vs. 17%, respectively). High DNA loads were noticed, indicating that this discrepancy could not be explained by mainly light infections missed by microscopy. In Mozambique, microscopy and PCR findings were highly comparable, showing a high prevalence for most of the helminth species tested. Based on PCR findings, behavioural risk factors were found to be associated with STH infections in Indonesia. Diagnosis based on DNA detection in stool seems a more reliable approach than stool microscopy for studying the distribution of helminth infections and to compare different target populations.

Keyword:
Soil-transmitted helminths, Indonesia, Mozambique, diagnostics, stool microscopy, real-time PCR, risk factors

INTRODUCTION

Soil-transmitted helminth (STH) infections are still a major global public health problem, despite many years of morbidity control through mass drug administration [1, 2]. It is estimated that currently approximately 1.4 billion people globally are infected with *Ascaris lumbricoides*, nearly 800 million people with *Trichuris trichiura*, and 740 million people with the hookworm species *Ancylostoma duodenale*, *A. ceylanicum* or *Necator americanus*. On top of that around 100 million people are estimated to be infected with *Strongyloides stercoralis*. This intestinal nematode species is often ignored, while its route of transmission is very similar to the hookworm species and infection is of significant clinical importance [3, 4]. All these soil-transmitted helminth infections affect, almost exclusively, the poorest populations within the tropics [1].

In Southeast Asia (SEA) numerous countries are endemic for STH infections and overall this region contributes approximately one-third of the global disease burden caused by these helminths [4]. In particular Indonesia, with an estimated 219 million infected cases, has the largest number of STH infected individuals within SEA [4]. Nevertheless, national figures about the precise distribution and prevalence show considerable scatter, with a reported prevalence of STH infections ranging from 10% to >50% and substantial gaps in the epidemiological data because certain regions of the archipelago are more intensely monitored than others [5, 6].

For the diagnosis of STH infections, faecal examination by microscopy is considered to be the "gold standard". The most widely applied microscopy method to identify the STH species as well as to quantify intensity of infection, is the Kato-thick smear (KS). This method is also recommended by the World Health Organization [5, 7]. Alternatively, the formol-ether concentration (FEC) method can be used. Although being more laboratory dependent, as it includes a centrifugation step, it has the major advantage of being the nearest to an all-round diagnostic procedure for the detection of a range of intestinal parasite species. To combine the advantages of each methods, several studies have applied both KS and FEC [8-11].

Although stool microscopy is a relatively simple diagnostic procedure, it poses many challenges, including the need to properly train microscopists and a lack of standardized protocols for collecting the sample, preparing the slide, performing the microscopic examination, and implementing external quality assessment schemes [12, 13]. This lack of standardization becomes most evident when comparing data collected at various study sites using different microscopy protocols [5, 14]. For example, the sensitivity of the detection of hookworm eggs can vary between microscopists, but is also largely depending on the number of stool samples collected, the number of microscopy examinations per stool samples and the overall intensity of infection within the population. In particular in populations where most individuals harbour light intensity of infection, some minor differences in the diagnostic protocol can have large consequences in the number of detected cases, and therefore on decisions to be made about the introduction or the continuation of helminth control programs [12, 13, 15].

Stool microscopy is also the most commonly used diagnostic procedure when analysing the risk factors that underlie helminth infections [16-20]. The prevalence of STH infections results from a complex interplay between multiple factors, including environmental settings and host related characteristics such as age, socioeconomic status, cultural habits, access to sanitation, and access to essential medication [1, 21-24]. Disentangling the relative contributions of each of the host-related risk factors associated with STH infections is relevant for a better understanding which socio-behavioural patterns influence infection and re-infection rates and for more insight into the beneficial effects of control strategies. The fact that most studies analysing risk factors associated

with STH infections are based on sub-optimal diagnostic procedures such as microscopy, might bias the overall conclusions.

Alternative diagnostic procedures have been developed in recent years, including methods to detect and quantify parasite DNA in stool samples using multiplex real-time PCR. These methods are both highly sensitive and highly specific [25-27]. Although real-time PCR has the practical limitation of requiring high-tech laboratory equipment, it can be standardized to a large extent, enabling researchers to pool and compare findings from various study populations [13]. Nevertheless, so far there is hardly any published data using the outcome of real-time PCR on stool samples collected in different countries to evaluate STH prevalence and intensity of infection, nor to specifically study risk-factors associated with STH infection [28].

In this study, we compared prevalence and infection intensity of STH between two unrelated community-based surveys, one conducted in Flores, Indonesia, the other in Beira, Mozambique. Part of the study data has been published before [29, 30]. Infection status was determined both by stool microscopy and by multiplex real-time PCR. Though similar approaches were used for the detection and quantification of parasite-specific DNA in stool, the microscopy procedures used at each of these two cross-sectional surveys differed substantially. Based on the outcome of the comparison, real-time PCR data was subsequently used to identify relevant risk factors associated with STH infections within the Indonesian study.

MATERIALS AND METHODS

Ethics statement

The two studies presented here have been approved by authorized ethic committees as described previously [29, 31, 32]. In Indonesia, the study protocol has been seen by the Faculty of Medicine of the Universitas Indonesia, in Mozambique by the Beira Committee of Medical Ethics and both protocols have been filed in the Netherlands by the Committee of Medical Ethics of the Leiden University Medical Center. Participants were informed about the objectives of the study prior to sample collection and all samples were anonymized before testing [29, 31, 32].

Indonesia: Study design, sample collection and microscopic examination

The Indonesian samples were collected as part of a larger double-blind randomized longitudinal study on the effect of treating inhabitants for two years with three monthly albendazole (400 mg) doses, looking at the effect of STH infections on malarial parasitemia and allergy. Study design and major outcomes have been described earlier [31-33]. The study was conducted in Nangapanda sub-district, a semi-urban area in Ende city, on Flores Island. This district consists of three villages, named Ndeturea, Ndorurea One and Ndorurea [31, 32]. Data regarding participant demographics (e.g., gender, age, parental education, occupation) and household properties (e.g., housing materials, floor materials, toilet, water supply) were collected by interviewing the individual participants. Parental education levels were categorized as "low" for parents who were illiterate or completed elementary school and "high" for parents who completed secondary school or higher. Because the vast majority of the population comprised of farmers, occupation was categorized as either "farmer" or "non-farmer".

A total of 1612 participants (including 510 children and 1102 adults) provided a stool sample during the 2008 dry season, from May through July. From each stool sample an aliquot of approximately one gram was stored at -20 $^{\circ}$C. The remaining stool was preserved in 4% formaldehyde, stored at room temperature, and transported to the Laboratory of Parasitology in Jakarta, Indonesia where microscopic examination was performed 2-3 month later. Following

formol-ether concentration (FEC), sediments were examined for the presence of eggs of STHs, including larvae of *Strongyloides stercoralis* [34]. The frozen aliquots were transported to the Netherlands, where DNA extraction and real-time PCR analysis was performed at each stool samples known to be matched with a blood sample for *Plasmodium* DNA detection [33]. In total 1087 samples were used for the current study, as 525 of the 1612 participants were excluded for the following reasons: (i) no paired blood sample was available; (ii) microscopy data was not available; or (iii) inhibition occurred during PCR amplification (Figure 1).

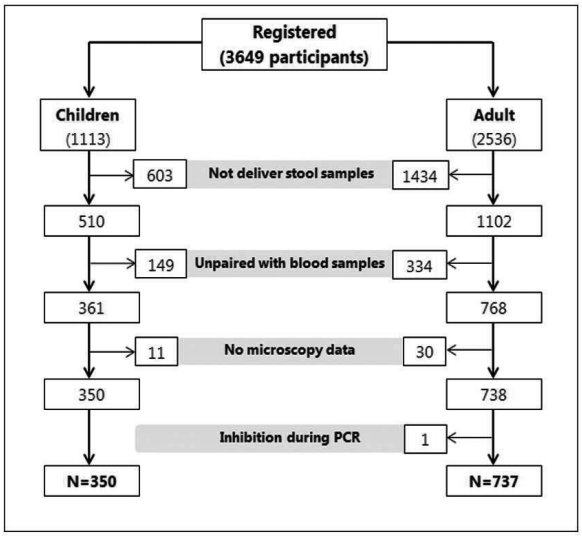

Figure 1. Flow-chart showing the sample selection of children and adults in the Nangapanda sub-district of Indonesia

Mozambique: Study design, sample collection and microscopic examination

The Mozambique samples were collected in Inhamudima, an informal settlement in Beira, Mozambique in order to study the distribution of intestinal parasites in the area. Similar to the Nangapanda, Indonesia population, study design and major outcomes have been describes previously [29]. In brief, a total of 303 stool samples were collected during a household randomized survey including 399 individuals from 63 households. The survey was performed during the dry season, June to August, of 2007. Within 24 hours after collection the samples were thoroughly examined for intestinal parasites at the laboratory of the University Hospital in Beira by a group of well-trained laboratory researchers. The microscopy methods included a direct smear, a formol-ether concentration (FEC) procedure, a single 25-mg Kato thick smear (KS) examination, a Baermann procedure and a copro-culture. Further details describing how these microscopy methods were applied within this study population have been described before [29].

The number of eggs or larvae present in each individual sample was recorded per helminth species, as well as per diagnostic test used. A stool aliquot of approximately 0.3 ml was mixed with 1 ml of 96% ethanol and transported to the Netherlands for DNA extraction and real-time PCR analysis.

DNA extraction and amplification

The procedure of DNA extraction and detection of parasite-specific DNA by real-time PCR was performed as described before [29, 32, 35]. In brief, DNA was isolated using DNeasy 96 Blood & Tissue Kit spin columns in accordance with the manufacturer's instructions (Qiagen, Hilden,

Germany). For the Mozambique samples this was done after removal of the ethanol via a washing procedure [36]. Real-time PCR was performed to detect the DNA from *Ancylostoma* spp. (including both *A. duodenale* and *A. ceylanicum*), *N. americanus*, *A. lumbricoides*, and *S. stercoralis* [29, 32, 35]. No assay was available yet for sensitive detection of *T. trichiura* DNA when the study samples were tested [37]. Negative and positive control samples for each species were included in each PCR run. A fixed amount of phocin herpes virus-1 (PhHV-1) was added within the isolation lysis buffer as an internal control and virus-specific primers and detecting probe were included in each reaction mixture. The PCR output consisted of a cycle threshold (Ct) value representing the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence. Samples were considered positive showing a Ct-value below 50 [26, 29, 32]. The internal control PhHV-1 was detected at the expected Ct-value in all tested samples, with the exception of one stool originating from Indonesia (Figure 1).

Statistical analysis

Data were analysed using SPSS 20.0 (IBM, Chicago, IL). Descriptive frequency analysis was used to describe the overall characteristics of the participants, as well as the prevalence of each STH species based on microscopy and real-time PCR methods. The acronym ANAS describes those STH species included in the PCR detection, namely *Ancylostoma* spp., *N. americanus*, *A. lumbricoides*, and *S. stercoralis*. A chi-square (X^2) test was used to analyse the association between ANAS infection rates obtained using microscopy and the infection rates obtained using real-time PCR. The Mann-Whitney U test was used to detect a difference in median Ct-values between microscopically diagnosed ANAS positive and negative samples. Intensity of infection was arbitrarily categorized for each DNA target into low DNA load ($35 \leq Ct < 50$), moderate DNA load ($30 \leq Ct < 35$), or high DNA load ($Ct < 30$), while negative PCR results were recoded as $Ct = 50$ [38].

Based on the demographic data availability from the Indonesia study, a risk factor analysis was performed for STH infections. Infection status, defined as positive for at least one of the ANA PCR targets, was used as the binary outcome of risk factor analysis in each group. Children (≤15 years of age) and adult (>15 years of age) were analysed separately. To assess the risk factors associated with STH infections, logistic regression analysis under Generalized Linear Mixed Models (GLMM) was used in order to take into account clustering by household. Univariate analysis was applied to measure the correlation between STH infections detected using real-time PCR (dichotomous outcome) and potential risk factors (e.g., age, gender, village, occupation, parental education level, house materials, floor materials, toilet, water resource, and main staple). In the multivariate model, variables were included that were determined to be significant in the univariate analysis and the variables age, gender, and village were included as *a priori* confounders. In both the univariate and multivariate models, the data is presented as an odds ratio with 95% confidence interval (CI). Differences with a *p*-value <0.05 were considered to be statistically significant.

RESULTS

Population characteristics

Detailed characteristics of a total of 1087 participants from the Indonesia study, including demographics and household information, are summarized in Table 1. The age of the participants ranged from 4 to 78 years, with a median age of 31 years; 41.3% of the participants are male. Of the children who reported their parents' education level, 52.8% and 39.9% reported at least secondary school for their father and mother, respectively. Slightly more than 50% of the adults

classified themselves as farmers. Most of the households' walls were constructed of wood (73.3%) and contained a ceramic-covered floor (80.5%). The majority of households were equipped with an indoor toilet (65.5%) and pipeline water availability (54%). Rice was the main food staple in the majority of households (78.4%); cassava was the main food staple in the remaining households (21.6%).

In the Mozambique study, the age of the 303 participants ranged from 1 to 72 years, with a median age of 17 years; 47.5% of the participants are male. Additional demographics and household data were not available.

Table 1. Overview of the demographics and household data of the 1087 participants from the Nangapanda sub-district of Indonesia

	Children, n (%)	Adults, n (%)
N	350	737
Gender (male)	176 (50.3)	273 (37.0)
Age (years)		
4-7	111 (31.7)	NA
8-10	115 (32.9)	NA
11-15	124 (35.4)	NA
16-26	NA	147 (19.9)
27-36	NA	135 (18.3)
37-46	NA	186 (25.2)
47-56	NA	132 (17.9)
≥ 57	NA	137 (18.6)
Village		
Ndeturea	64 (18.3)	159 (21.6)
Ndorurea One	148 (42.3)	324 (44.0)
Ndorurea	138 (39.4)	254 (34.5)
Occupation (farmer)[1]	N/A	363 (50.6)
Father's education (high)[2]	118 (52.8)	ND
Mother's education (high)[3]	112 (39.9)	ND
Household data		
N	345	735
Wall material (stone)	82 (23.8)	206 (28.0)
Floor material (ceramic)	268 (77.7)	601 (81.8)
Water resource (piped)	182 (52.8)	401 (54.6)
Toilet (indoor)	216 (62.6)	491 (66.8)
Main food staple (rice)	276 (80.0)	571 (77.7)

[1] Data were available for a total of 717 participants
[2] Data were available for a total of 246 participants
[3] Data were available for a total of 281 participants
NA: not applicable; ND: not determined

Prevalence of STH infections in the populations

Table 2 summarizes the prevalence of each helminth species, or combination of species, detected either by microscopy or by real-time PCR, for both study populations, while Figure 2 focusses solely on those helminth species included in the multiplex real-time PCR and also depicts the relative parasite species–specific DNA loads measured.

Indonesia

In the Indonesia study, 47% (n=511) of the total study population were diagnosed with one or more STH species by using microscopy. Eggs of *A. lumbricoides*, hookworm, and *T. trichiura* were identified in 25.1, 16.7, and 27.5% of the samples, respectively (Table 2). No *S. stercoralis* larvae were seen. Multiple STH species were detected in 39.7% of the participants. In 163 (31.9%)

of the 511 STH-positive cases two different species were identified, with *A. lumbricoides* together with *T. trichiura* being the most common combination (n=102). In 2.5% of the positive cases three STH species were detected.

Real-time PCR revealed *N. americanus* DNA and *Ancylostoma* spp. DNA in 71.8% and 5.3% of the stool samples, respectively (Figure 2B). *A. lumbricoides* DNA was detected in 35.1% of cases, and *S. stercoralis* DNA was detected in 1.7% of cases (Figure 2B, Table 2). Multiple infections were detected in 396 (44.7%) of the 885 PCR positive stool samples, with the majority (n=391) harbouring a co-infection of two species. In total 81.4% of the samples were positive for at least one of the ANAS targets tested at the multiplex real-time PCR. This percentage is 2.3 fold higher than the percentage of cases diagnosed by microscopy as being positive for the same species (35.4%) (Table 2).

Slightly more than 60% of cases that were positive for *N. americanus* or *A. lumbricoides* based on real-time PCR analysis were categorized as having a high DNA load (Figure 2B). With respect to *Ancylostoma* spp., slightly fewer than 45% of the positive cases had a high species-specific DNA load. The positive cases of *S. stercoralis* were categorized primarily as having a low DNA load (n=9).

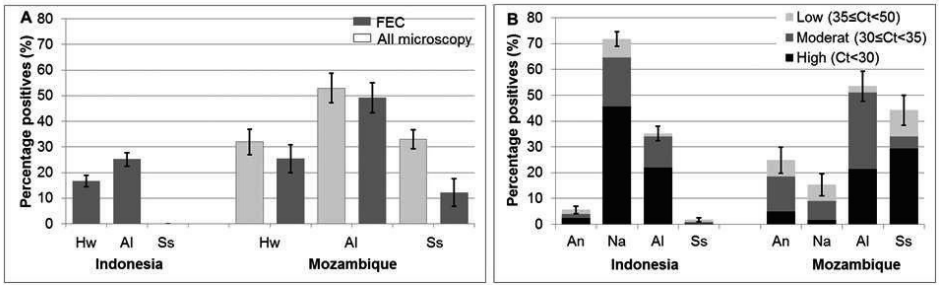

Figure 2. Summary of the percentage of positive STH infections in Indonesia (N=1087 participants) and Mozambique (N=303 participants) studies for the indicated soil-transmitted helminths (STHs)

(A) STH infections were measured using the formol-ether concentration (FEC) or a combination of five microscopy methods (direct smear, Kato-Katz, FEC, the Bearmann method, and copro-culture). (B) DNA load was measured using real-time PCR and stratified according to cycle threshold (Ct) value. Whiskers in each bar indicate 95% confidence intervals of the observed prevalence Hw= Hookworm; Na= *N. americanus*; An= *Ancylostoma* spp.; Al= *A. lumbricoides*; Ss= *S. stercoralis*.

Mozambique

In the Mozambique study, the FEC method revealed *A. lumbricoides*, hookworm, *S. stercoralis*, and *T. trichiura* infections in 49.2, 25.4, 12.2, and 90.8% of samples, respectively. Combining the outcome of all five microscopy procedures resulted in 296 positive cases (96.0%) for at least one of the target parasites, which is less than 3% higher than the 284 cases diagnosed using FEC only. The highest difference was seen for *S. stercoralis*, where the combined outcome of the five different microscopy procedures resulted in the detection of 63 additional cases (Table 2). Multiple helminth species were detected in 57.5% of the STH-positive cases. Primarily *A. lumbricoides* in combination with *T. trichiura* (n=69) was seen in the 115 cases with double infections. In the remaining multiple species-positive cases three (n=31) or four (n=33) helminths species were detected.

Real-time PCR detected *N. americanus* DNA in 15.2% of cases and *Ancylostoma* spp. DNA in 24.8% of cases (Figure 2B) and 5% (n=15) of the total study population showed a mixed infection with both species. *A. lumbricoides* DNA was detected in 53.5% of cases, and *S.*

stercoralis DNA was detected in 44.2% of cases (Figure 2B, Table 2). The overall percentage of STH infections detected using real-time PCR was 77.9% (n=236 positive cases), with multiple STH species in 129 cases, the majority of which were double infections (n=92).

Approximately half of the cases positive by PCR for *Ancylostoma* spp., *A. lumbricoides*, or *N. americanus* showed a Ct-value between 30 and 35 (moderate DNA load). Only for the *S. stercoralis* PCR 66.4% of the positive cases showed a Ct-value below 30, indicating a high DNA load.

Table 2. Positive number and percentage of soil-transmitted helminth (STH) infections determined using the indicated diagnostic methods in the Indonesia and Mozambique studies

	Indonesia (N=1087)			Mozambique (N=303)			
	FEC	Real-time PCR	All Techniques	FEC	All Microscopy[1]	Real-time PCR	All Techniques
Hookworms[2]	182 (16.7)	788 (72.5)	796 (73.2)	77 (25.4)	98 (32.3)	106 (35.0)	115 (38.0)
A. lumbricoides	273 (25.1)	381 (35.1)	415 (38.2)	149 (49.2)	161 (53.1)	162 (53.5)	169 (55.8)
S. stercoralis	0 (0.0)	19 (1.7)	19 (1.7)	37 (12.2)	100 (33.0)	134 (44.2)	146 (48.2)
Overall ANAS[3]	385 (35.4)	885 (81.4)	886 (81.5)	190 (62.7)	223 (73.6)	236 (77.9)	245 (80.9)
T. trichiura	299 (27.5)	ND	299 (27.5)	275 (90.8)	282 (93.1)	ND	282 (93.1)
Overall STH[4]	511 (47.0)	885 (81.4)	933 (85.8)	284 (93.7)	296 (96.0)	236 (77.9)	293 (96.7)

[1] All microscopy is the combined results from direct smear, Kato-Katz, FEC, the Baermann method, and copro-culture, see materials and methods section for further details
[2] *N. americanus* and *Ancylostoma spp*
[3] ANAS: *Ancylostoma* spp., *N. americanus*, *A. lumbricoides* and *S. stercoralis*
[4] Includes all soil-transmitted helminths examined in this study
ND: not determined

Parasite species–specific DNA load compared to the microscopy results

Indonesia

In the Indonesia study population, the highest level of discrepancy between PCR and microscopy outcome was seen for the hookworm species, with 614 samples (56.5%) in which hookworm DNA was detected while microscopy was negative and 8 samples (0.7%) where hookworm eggs were seen, while the PCR was negative. Those 8 microscopy positive samples all showed low egg counts, the maximum number of eggs observed in the sediment was four. The 614 samples diagnosed by PCR only, included samples with high DNA loads.

For *A. lumbricoides* the number of samples positive by PCR only was lower (142; 13.1%), but at the same time the number of samples positive by microscopy only was higher (34; 3.1%) in comparison to the hookworm. Again eggs counts were relatively low in those samples tested negative with the PCR, with a maximum of 68 eggs counted in the sediment. No significant difference was found in the Ct-value between the *A. lumbricoides* egg-negative stool samples (n=142, median Ct-value 29.2, with a range of 22.9-37.6) and the *A. lumbricoides* egg-positive stool samples (n=239, median Ct-value 28.6, with a range of 20.2-43.0) as determined by microscopy. In total 15 cases (1.4%) were positive by microscopy for at least one of the ANAS targets, while the PCR was negative for all targets tested.

Mozambique

In the Mozambique study population, in total 9 cases (3.0%) were positive by microscopy for at least one of the ANAS targets, while the PCR was negative for all targets. The highest level of discrepancy between PCR and microscopy outcome was seen with *S. stercoralis*, with 46 samples (15.2%) in which *S. stercoralis* DNA was detected while microscopy was negative and 12 samples (4.0%) in which L3 larvae were seen, while the PCR was negative. Each of those 12 microscopy positive samples were positive in only one of the five microscopy techniques used and the number of larvae were low. Within the group of *S. stercoralis* PCR positive samples, DNA loads were significantly higher in microscopy positive samples (n= 88, median Ct-value 26.2) in comparison to microscopy negative samples (n= 46, median Ct-value 34.4, $p<0.001$).

Risk factors associated with helminth infections in children and adults

Table 3 summarizes the putative risk factors for the helminth infections detected by the ANAS-PCR, stratified by the child and adult groups in the Nangapanda sub-district of Indonesia. A univariate analysis revealed that age, village, parental education level, and wall materials were all potentially factors associated with ANAS infection in children. With respect to age, children aged 8-10 years old had a higher risk to be infected with ANAS compared to those aged 4-7 years old (OR: 2.25; 95% CI: 1.06-4.76; $p=0.034$). Table 3 also showed that, living in the village of Ndeturea, living in Ndorurea One (OR: 0.24; 95% CI: 0.07-0.76; $p=0.017$) or Ndorurea (OR: 0.18; 95% CI: 0.05-0.63; $p=0.007$) significantly reduced the likelihood of having an ANAS infection. Children whose fathers had a low level of education had a significantly higher risk of ANAS infection than children whose fathers had a high level of education (OR: 2.29; 95% CI: 1.18-4.46; $p=0.015$); in contrast, the mother's education level had no effect on the child's likelihood of having an ANAS infection (OR: 1.55; 95% CI: 0.71-3.38; $p=0.267$). Finally, children who lived in a house built with bamboo or wood as the wall material had a significantly higher risk of having an ANAS infection compared to children who lived in a house with stone walls (OR: 2.82; 95% CI: 1.38-5.74; $p=0.005$).

In the adult group univariate analysis revealed that village, occupation, and all house components were positively associated with having ANAS infection. However, in multivariate analysis, only occupation (OR: 2.44; 95% CI: 1.57-3.81; $p<0.001$), the water resource (OR: 1.60; 95% CI: 1.04-2.46; $p=0.034$), and having an indoor toilet (OR: 2.51; 95% CI: 1.48-4.26; $p=0.001$) remained positively associated with ANAS infection (Table 3).

DISCUSSION

Recent estimates suggest that approximately 5.3 billion people worldwide are at risk of developing a chronic STH infections, with 69% living in Asia [4, 5, 22]. However, these estimates are based primarily on diagnostic procedures that have known limitations. Microscopic stool examination lacks sensitivity, meaning that light to moderate infection intensities are easily missed. In addition, this method is highly observer-dependent and poorly standardized. Because of these limitations, improving diagnostic methods has received high priority on the research agenda of helminth-related diseases in humans [14, 15, 39].

In the present study, the use of real-time PCR as a diagnostic tool for STH was further evaluated by comparing two distinct endemic communities, one in Ende, Indonesia, the other in Beira, Mozambique. In these two regions, the prevalence and intensity of four helminth species was measured both by microscopy and real-time PCR. Microscopy-based diagnostics was performed in accordance with the most feasible procedures within the given local setting, while a

Table 3. Risk factors associated with STH infections detected using real time PCR in the children and adults the Indonesia study

Indicators		Children (N=350)					Adults (N=737)				
		n (% STH positive)	Unadjusted		Adjusted[1]		n (% STH positive)	Unadjusted		Adjusted[1]	
			OR (95% CI)	p-value	OR (95% CI)	p-value		OR (95% CI)	p-value	OR (95% CI)	p-value
Gender	Female	174 (75.9)	1	0.616			464 (83.0)	1	0.654		
	Male	176 (79.0)	1.14 (0.68-1.90)				273 (83.9)	1.10 (0.73-1.65)			
Village	Ndeturea	64 (92.2)	1		1		159 (93.7)	1		1	
	Ndorurea One	148 (79.1)	0.34 (0.13-0.90)	**0.030**	0.24 (0.07-0.76)	**0.017**	324 (79.9)	0.30 (0.15-0.59)	**0.000**	0.54 (0.26-1.09)	0.085
	Ndorurea	138 (68.8)	0.20 (0.08-0.52)	**0.001**	0.18 (0.05-0.63)	**0.007**	254 (81.1)	0.32 (0.16-0.64)	**0.001**	0.58 (0.28-1.23)	0.156
Age (years)	4-7	111 (68.5)	1		1						
	8-10	115 (80.9)	2.06 (1.09-3.86)	**0.025**	2.25 (1.06-4.76)	**0.034**			NA		
	11-15	124 (82.3)	2.237 (1.20-4.18)	**0.012**	1.87 (0.85-4.13)	0.122					
	16-26						147 (87.7)	1			
	27-36						135 (78.5)	1.90 (1.00-3.63)	0.051		
	37-46			NA			186 (81.7)	1.50 (0.81-2.81)	0.199		
	47-56						132 (82.6)	1.38 (0.70-2.70)	0.353		
	≥57						137 (86.1)	1.10 (0.55-2.20)	0.797		
Occupation	Non-farmer			NA			354 (76.3)	1	**0.000**	1	**0.000**
	Farmer						363 (90.4)	2.85 (1.86-4.35)		2.44 (1.57-3.81)	
Father Education	High	118 (65.3)	1	**0.001**	1	**0.015**			NA		
	Low	128 (85.2)	3.00 (1.61-5.60)		2.29 (1.18-4.46)						
Mother Education	High	112 (66.1)	1	**0.002**	1	0.267			NA		
	Low	169 (82.8)	2.54 (1.43-4.51)		1.55 (0.71-3.38)						
Wall Materials	Stone	82 (64.6)	1	**0.001**	1	**0.005**	206 (77.7)	1	**0.021**	1	0.679
	Bamboo	263 (82.1)	2.68 (1.52-4.73)		2.82 (1.38-5.74)		529 (85.6)	1.63 (1.08-2.45)		1.10 (0.70-1.72)	
Floor Materials	Ceramic	268 (76.9)	1	0.752			601 (81.7)	1	**0.020**	1	0.321
	Mud	77 (81.8)	1.11 (0.57-2.17)				134 (91.0)	2.12 (1.13-4.00)		1.40 (0.72-2.71)	
Water Resource	Piped	182 (76.9)	1	0.569			401 (80.3)	1	**0.005**	1	**0.034**
	Non-Piped	163 (79.1)	1.16 (0.69-1.96)				334 (87.1)	1.81 (1.20-2.74)		1.60 (1.04-2.46)	
Toilet	Indoor	216 (76.4)	1	0.118			491 (79.4)	1	**0.000**	1	**0.001**
	Outdoor	129 (80.6)	1.57 (0.89-2.78)				244 (91.4)	2.75 (1.67-4.55)		2.51 (1.48-4.26)	
Main Staple	Rice	276 (74.6)	1	0.135			571 (80.6)	1	**0.000**	1	0.118
	Cassava	69 (91.3)	2.21 (0.78-6.27)				164 (93.3)	3.05 (1.63-5.68)		1.71 (0.87-3.30)	

[1] Adjusted by age, gender, and village
NA: not applicable

standardized real-time PCR method was used for all samples at a centralized laboratory. Unfortunately it was not possible to evaluate the use of real-time PCR for *T. trichiura* infections, as at that time no suitable method existed for extracting DNA from *T. trichiura* eggs [37].

Interestingly, significant differences were seen between the two study populations when comparing the outcome of each diagnostic procedure, in particular for hookworm infections. In the Indonesian study population hookworm eggs were detected in 16.7% of stool samples by using the FEC method, whereas real-time PCR analysis revealed the presence of hookworm DNA in the faeces of 72.5% of the participants. On the other hand, in in the Mozambique study population, hookworm eggs and DNA were detected in 25.4% and 35.0% of the participants, respectively. One possible explanation for this difference in outcomes could be the length of time elapsed between collecting the stool sample and performing the FEC procedure. In the Indonesian study, the preserved samples had to be sent to the local central laboratory, and the FEC examination was performed 2-3 months after sample collection. This is very different from the Mozambique situation where the FEC procedure was performed within 24 hours of sample collection. A delay between sample collection and specimen processing in the laboratory can affect the diagnostic performance of the FEC procedure, particularly for hookworm species [40]. In the Mozambique setting, four additional microscopy procedures were used besides the FEC. This somewhat increased the number of hookworm positive samples from 25.4% to 32.3%, a number which is still lower than de 35.0% of samples positive by PCR. Of all parasite species detected, *S. stercoralis* was the only one which showed a pronounced increase in the number of positive cases when all five techniques were used. This because two of the five techniques applied are specifically designed for the detection of *S. stercoralis* larvae, namely the Baermann method and the copro-culture. Previous studies already showed that single-method sampling fails to detect *S. stercoralis* larvae in as many as 70% of known positive cases [41].

Although overall PCR was found to be far more sensitive than microscopy, both study populations showed approximately 1-3% of the cases to be positive for at least one helminth species by microscopy while the ANAS PCR was negative for all targets. These so called false negative real-time PCR cases seem to occur more often when the intensity of infection, i.e. based on microscopy, is low. Most likely this is due to the fact that the DNA detection procedure is actually based on very small volumes. When intensity of infection is too low, parasite's eggs and free DNA might simply be lacking within the aliquot actually used for DNA isolation. In addition, the possibility of human error cannot be completely excluded, e.g. some structures of for instance plant-origin found in the faecal smear might have been erroneously identified as hookworm or *Ascaris* eggs [12].

In both studies only a single sample has been collected per participant. It has often been stated that examinations based on one stool sample only is inadequate, and at least three stool samples are required to increase the negative predictive value above 90% [42, 43]. However, in order to obtain more than one stool sample per participant, a range of practical and logistical issues need to be solved. A substantial number of people cannot deliver daily and in many cultural situations study subjects, in particularly teenagers, are embarrassed to provide a stool sample. The number of dropouts is also likely to increase if consecutive stool samples are requested. In order to diagnose *T. trichiura*, hookworm, and *S. stercoralis* infections accurately even as many as 20 stool samples should be examined in a low-intensity setting. With the exception of specialized, small-scale studies, such a higher sample number is totally not feasible [42]. Therefore, diagnostic methods that provide high sensitivity and have low technical demands are urgently needed.

A relatively new microscopy-based diagnostic method is the FLOTAC [9, 44, 45]. Several studies have been performed to evaluate this technique and found that FLOTAC can be more sensitive than both the Kato-smear and FEC methods at detecting STH eggs [9, 44-46]. However, the FLOTAC has the limitation it is a time-consuming procedure which also requires a large bucket centrifuge with a special adapter. The more recently introduced mini-FLOTAC method is a simplified version of the FLOTAC, which in addition allows the analysis of preserved stool samples. On the other hand, recent studies have shown that similar to the FEC procedure long-term storage of the faecal material in formalin decreases the diagnostic sensitivity of the mini-FLOTAC [47].

Hookworm infections were found to be abundant in both our study populations. Due to morphological similarities of the eggs, microscopy methods such as FEC and KS cannot be used to differentiate the hookworm species. Distinguishing between the two most common human hookworm species is important, as previous studies demonstrated that *Ancylostoma* spp. (i.e. *A. duodenale*) but not *N. americanus*, is independently associated with severe anaemia and iron deficiency [48]. Here we showed *Ancylostoma* spp. (including both *A. duodenale* and *A. ceylanicum*) to be more prevalent in the Mozambique study population, whereas *N. americanus* was considerably more prevalent in Indonesia.

High levels of polyparasitism were observed both in Nangapanda, Indonesia and in Beira, Mozambique. But different STH species predominated in each study. *N. americanus* was the most prevalent species in Nangapanda, Indonesia, whereas *A. lumbricoides* was the most prevalent species in Beira, Mozambique. Differences in the geo-climate might account for this finding, as surface temperature, altitude, soil type, and rainfall can strongly affect the distribution of STHs [1]. It is not known to what level the success of MDA is depending on the level of polyparasitism or on certain helminth species being predominant, but the increased usage of more sensitive diagnostic techniques, such as those based on DNA detection, will most likely provide more clarity on these issues [15].

Because real-time PCR seems to provide more accurate diagnostic data, PCR outcomes were used to analyse the risk factors associated with STH infections within the Indonesian study population. A correlation was found between the prevalence of STH and the materials used to construct the walls of the houses. However, this correlation was only seen in children and not in adults. Given that the better wall material was stone, this could have prevented environmental contamination from STH infections among the children but not the adults. The possible explanation could be that because more than half of the adults are farmers, the adult infection rate would not be as strongly affected by the type of material used to build their house. In addition, the father's level of education was found to be correlated with an decreased risk of STH infection in children, while no effect was seen for the mother. This is in contrast with several studies reporting a beneficial effect of the mother's level of education [49, 50]. Nevertheless, our study highlights the importance of obtaining information regarding the education level of both the mother and father in order to determine which had the higher influence. For example, in this study, the mothers do not always have an active role in caring for the children. This difference might also be related to the local culture. For example, a study in Latin America showed that men play a larger role in all aspects of life [21]. Similarly, in Nangapanda, Indonesia, men have a more active role in both the family and the community.

Working as a farmer highly increases one's risk of developing an STH infection [28], as reported recently in rural Bali, Indonesia [51]. Farmers are exposed to the soil almost continuously, which by definition is the way of transmission for soil-transmitted helminths. Several published

studies have reported that sanitary facilities such as water resources and an indoor toilet are strongly correlated with STH transmission, while good sanitation conditions lower the risk of STH infections [1, 24, 50, 52, 53]. Consistent with this correlation, we found that supplying water to the house through a pipeline and having an indoor toilet contributed to a significant decrease in STH infections in the adult group. On the other hand, even though schools can provide improved sanitation conditions, school children are at high risk for STH infections, as they play outdoors a large part of the day in high-risk settings such as rivers and the school yard [24, 52].

In conclusion, our findings confirm that real-time PCR is a sensitive method, suitable for high-throughput population-based screening for helminth infections. By using real-time PCR analysis in a highly standardized way at a centralized laboratory facility, it becomes feasible to compare STH infection rates between distinct study populations even when different microscopy procedures have been used in the field. Being a more reliable technique, DNA-based diagnostic methods are also more suitable to determine and evaluate potential risk factors associated with STH infections.

ACKNOWLEDGMENTS

We thank the participants from the Nangapanda-Flores region in Indonesia and the Inhamudima-Beira region of Mozambique. We also thank the Helminthology Group in the Department of Parasitology, Medical University, Indonesia; Natalie Vinkeles for helpful comments on earlier versions of the manuscript; and the students who were involved in the Indonesia and Mozambique studies. This study was funded by the Prof Dr P. F. C. Flu Foundation and The Royal Netherlands Academy of Arts and Science (KNAW), Ref 05-PP-35. The authors would also like to thank The Indonesian Directorate General of Higher Education (DIKTI)-Leiden University for providing a PhD scholarship.

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