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A Pentaplex Real-Time Polymerase Chain Reaction Assay for Detection of Four Species of Soil-Transmitted Helminths

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Abstract. Soil-transmitted helminth infections remain a major public health burden in low- and middle-income countries. The traditional diagnosis by microscopic examination of fecal samples is insensitive and time-consuming. In this study, a pentaplex real-time polymerase chain reaction (PCR) was evaluated for the simultaneous detection of *Ancylostoma*, *Necator americanus*, *Ascaris lumbricoides*, and *Strongyloides stercoralis*. The results were compared with those obtained by conventional parasitological diagnostic methods. Real-time PCR was positive in 48 of 77 samples (62.3%) and microscopic examination was positive in six samples (7.8%) only ($P < 0.05$). In conclusion, the real-time PCR assay described in this study provides a specific and sensitive diagnostic tool for the detection of these four helminth species in epidemiological studies and monitoring of treatment programs.

INTRODUCTION

Infections with soil-transmitted helminths (STH) *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworms (*Ancylostoma duodenale* and *Necator americanus*) are acknowledged as neglected tropical diseases. They result in a huge public health burden, affecting more than one billion people worldwide.[1–3] Infections with *Strongyloides stercoralis,* another STH, which affects 30 to 100 million people worldwide are probably even more underestimated.[4]

The effects of intestinal helminth infections depend on several factors such as the helminth species, intensity of the infection, and the host immunological status. These infections can result in chronic effects on health and nutritional status of the host, especially among children and immunocompromised individuals. Anemia, malnutrition, and gastrointestinal or pulmonary complaints are some of the problems associated with intestinal helminth infections.[5–7] In addition, fatal cases are seen with autoinfection by *S. stercoralis* because it may result in hyperinfection.[8]

Microscopic examination of stool samples is commonly used to detect infections with gastrointestinal helminths. Microscopy, however, is not sensitive and may result in misdiagnosis leading to delayed or inadequate treatment.[9] Commercial antibody detection tests are available for some STH infections, but are generally not sensitive or specific and are not able to differentiate current and past infections.

Conventional and real-time polymerase chain reaction (PCR) have proved to be highly sensitive and specific for detection of microbial agents and enteric pathogens.[9–15] Real-time PCR has proved to be highly sensitive and 100% specific in detecting *S. stercoralis* and hookworm infections as compared with microscopy.[9,15] Hence, real-time PCR of several helminth species provides a worthwhile diagnostic alternative because it allows multiplex detection and quantification while it decreases the chances of human error and DNA contamination as compared with conventional PCR. Moreover, it will save labor time and costs because gel electrophoresis is not needed.

In this study, a multiplex real-time PCR STH assay for the simultaneous detection of *Ancylostoma, N. americanus, A. lumbricoides,* and *S. stercoralis* was evaluated and compared with traditional parasitological techniques.

MATERIALS AND METHODS

Sample collection. Seventy-seven fecal samples were collected from patients presenting with abdominal symptoms in two district hospitals in Sarawak, Malaysia between November 2007 and January 2010. Informed consent was obtained from each patient according to the requirements of the ethical committees of Universiti Sains Malaysia and Ministry of Health, Malaysia. Anthelmintic treatment was provided based on the microscopy results as obtained in the local diagnostic laboratories.

Parasitological examination of fecal samples. In the hospital diagnostic laboratories, direct wet smear and Kato Katz[16] were performed within 4 hours after collection. For each sample, three slides of wet smears and one Kato-Katz preparation were examined by a trained microscopist.

One aliquot of each sample was placed in Parasafe preservative (Scientific Device Laboratories, Inc., Des Plaines, IL) and another aliquot in 1.5 mL screw-cap tube, which was stored frozen at −20°C. These two aliquots were shipped to USM where all the slides were examined using direct smear of the preserved samples (at least two slides per sample). In addition, zinc sulphate flotation[17] was performed on the preserved samples, whereby at least two slides of each surface and sediment samples were examined. If there was any discrepancy of the microscopy results, the data from USM was used because it was performed by a very senior and experienced microscopist. The frozen sample was processed for DNA isolation and real-time PCR. Samples were considered positive if the organism was detected by any of the three methods.

DNA extraction. The DNA was extracted using the QIAamp Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with minor modifications.[10] Briefly, ~100 mg of feces were suspended in 200 μL phosphate buffered saline (pH 7.2) containing 2% polyvinylpolypyrolidone (PVPP; Sigma, Steinheim, Germany). After heating for 10 minutes at 100°C, the suspension was treated with ATL buffer containing proteinase K for 3 hours at 55°C

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before performing the DNA extraction. Phocine herpesvirus 1 (PhHV-1) at 10^3 PFU/mL was added to the AL lysis buffer to serve as the internal control for the extraction process. The PhHV-1 was a kind contribution from Dr. Martin Schutten, Erasmus MC, Department of Virology, Rotterdam, The Netherlands.

Cloning of *Ancylostoma*, *N. americanus*, *A. lumbricoides,* and *S. stercoralis* target region. The PCR amplifications of the specific target regions of each organism (GenBank accession nos., AJ001594, AJ001599, AJ000895, AF279916) were performed using the primers as shown in Table 1. The PCR mixture (25 µL) contained PCR buffer (HotStar Taq master mix; Qiagen, Hilden, Germany), 5 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin (Sigma Aldrich Corp., St. Louis, MO), 300 nM of each species-specific primer, and 5 µL of the DNA from each native organism. The DNA amplification was performed for 15 minutes at 95°C followed by 50 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C using a conventional thermocycler (BioRad, Hercules, CA). Subsequently, the PCR product was cloned into pCR 2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA). After the transformation and screening of the clones, selected positive clones were sent for sequencing, and analyzed using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) to verify the presence of the target sequence in the recombinant plasmids. Serial 10-fold dilutions of the recombinant plasmids were subsequently used for construction of a standard curve for the real-time PCR assay.

Optimization of real-time multiplex PCR assay. Optimal primer concentrations for each organism were determined separately. The DNA amplification results of serial 10-fold dilution series of the plasmids from each pathogen were compared in separate and multiplex reactions. Subsequently, serial dilutions of each target were tested with and without the presence of internal control DNA to determine the influence of the latter on the assay. Each dilution series was also tested with and without the other target DNAs to assess the assay's ability to detect mixed infections. The amplification program consisted of 15 minutes at 95°C, followed by 50 cycles of 9 seconds at 95°C, and 60 seconds at 60°C. Amplification, detection, and data analysis were performed with the Rotor Gene 6000 (Rotorgene-Q, Hilden, Germany) real-time analyzer. Fluorescence was measured during the annealing step of each cycle. Fluorescence emitted at 555, 510, 610, 710, and 660 nm was measured for *Ancylostoma*, *N. americanus*, *A. lumbricoides*, *S. stercoralis*, and PhHV-1, respectively. The cycle threshold (Ct) value is defined as the number of PCR cycles required for the detection of fluorescence signal of the amplified products to exceed the set threshold value. As a consequence, higher quantities of DNA will result in lower Ct values and vice versa.

Real-time PCR amplification and detection. Table 1 shows the sequences of the primers and probes used in this study, which have shown 100% specificity when tested against DNA controls derived from a wide range of intestinal microorganisms.[9,15] To accommodate the specific fluorophore combination of the Rotor Gene 6000 real-time PCR system (Rotorgene-Q) the *A. lumbricoides*-specific probe was labeled with reference dye (ROX) and the *S. stercoralis*-specific probe was labeled with Alexa 680. The amplification reaction mixture (25 µL) consisted of PCR buffer (HotStar Taq master mix; Qiagen), 5 mM $MgCl_2$ (MBI Fermentas Inc., Amherst, NY), 0.1 mg/mL bovine serum albumin (Sigma Aldrich Corp.), 5 µL of template DNA, optimized concentrations of each species-specific primers (see Results), and 250 nM of each probe. Included in each PCR run are negative control comprising PCR mixture without DNA template; five positive controls comprising DNA of each helminth, and PhHV-1 DNA. The viral DNA internal control ensures there is no inhibition during the DNA extraction and PCR reaction. Amplification reactions were repeated for samples with Ct values above 35. Real-time PCR assay was considered as negative when the Ct value was more than 40 or no amplification curve was obtained. In addition, samples with a Ct value above 37 from the PhHV-1 internal control amplification are considered to be hampered by fecal inhibitory factors.

Preparing of the master mix, extraction of DNA, and handling of PCR products were all performed in separate

TABLE 1

Oligonucleotide primers and detection probes of real-time polymerase chain reaction (PCR) for detection of *Ascaris lumbricoides, Strongtloides stercoralis, Ancylostoma, Necator americanus,* and phocine herpesvirus-1 as an internal control

Target organism	Oligonucleotide name	Oligonucleotide sequence	Size of target region	Target gene	Ref.
A. lumbricoides	Alum96F	5′-GTA ATA GCA GTC GGC GGT TTC TT-3′	89 bp	ITS1	18
	Alum183R	5′-GCC CAA CAT GCC ACC TAT TC-3′			
	Alum124T	ROX-5′-TTG GCG GAC AAT TGC ATG CGA T-3′ -black hole quencher 2			
S. stercoralis	Stro18S-1530F	5′-GAA TTC CAA GTA AAC GTA AGT CAT TAG C-3′	101 bp	18S	15
	Stro18S-1630R	5′-TGC CTC TGG ATA TTG CTC AGT TC-3′			
	Stro18S-1586T	Alexa 680-5′-ACA CAC CGG CCG TCG CTG C-3′ -black hole quencher 3			
Ancylostoma	Ad125F	5′-GAA TGA CAG CAA ACT CGT TGT TG-3′	71 bp	ITS2	9
	Ad195R	5′-ATA CTA GCC ACT GCC GAA ACG T-3′			
	Ad155MGB	VIC-5′-ATC GTT TAC CGA CTT TAG-3′ -nonfluorescent quencher			
N. americanus	Na58F	5′-CTG TTT GTC GAA CGG TAC TTG C-3′	101 bp	ITS2	9
	Na158R	5′-ATA ACA GCG TGC ACA TGT TGC-3′			
	Na81MGB	FAM-5′-CTG TAC TAC GCA TTG TAT AC-3′ -nonfluorescent quencher			
Phocine herpesvirus	PhHV-267s	5′-GGG CGA ATC ACA GAT TGA ATC-3′	69 bp	gB	19
	PhHV-337as	5′-GCG GTT CCA AAC GTA CCA A-3′			
	PhHv-305tq	Cy5-5′-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-3′-black hole quencher 2			

rooms using allocated pipettes and equipment to prevent contamination.

Statistical analysis. Statistical analysis was performed by using SPSS software (version 12, SPSS Inc., Chicago, IL) and MedCalc (http://www.medcalc.be). The analysis of the detection rates of parasitological diagnosis and the pentaplex real-time PCR assay was analyzed using the chi-square (χ^2) test or Fisher's exact test. A P value < 0.05 was considered significant.

RESULTS

Optimization of the real-time PCR assay and background test. The optimized primer concentrations obtained were as follows: 40 nM for each *Ancylostoma* and *A. lumbricoides,* 60 nM for *S. stercoralis,* 80 nM for each *N. americanus*, and PhHV-1. The Ct values obtained from testing the dilution series of each pathogen in the singleplex assays and in the multiplex assay were similar and the same analytical sensitivity was achieved. The presence of DNA from the other targets as background or DNA from the internal control did not change the individual performance of the assays.

Detection limits of recombinant DNA (rDNA) copy numbers. The detection limits using the appropriate recombinant DNA (rDNA) were determined to be 10 copies for *A. duodenale*, *A. lumbricoides*, and *S. stercoralis*, and 10^3 copies for *N. americanus*.

Comparison of microscopy and real-time PCR. In all PCR runs, the controls (negative, positives, and PhHV-1) performed as expected; no amplification curve was seen in negative controls and amplification of the internal control (PhHV-1), and positive controls were detected in all PCR reactions. In addition, duplicate results for samples showing a Ct value above 35 were consistent. All amplifications of PhHV-1 internal control gave Ct values below 37, indicating absence of inhibitory substances. Table 2 shows the detection of single and multiple infections using microscopy and real-time PCR, respectively.

TABLE 2

Comparison of microscopy (Kato-Katz thick smear, zinc sulphate flotation technique and/or direct wet smear) and real-time multiplex polymerase chain reaction (PCR) assay of soil-transmitted helminth (STH) in fecal samples (N = 77)

Organism	Parasitological techniques	Real-time multiplex PCR
	Single infection	
Hookworm;	1	
1. *Necator americanus*		8
2. *Ancylostoma*		6
Ascaris lumbricoides	3	2*
Strongyloides stercoralis	2	15
	Multiple infections	
S. stercoralis and *N. americanus*	0	8
S. stercoralis and *Ancylostoma*	0	3
S. stercoralis, N. americanus and *Ancylostoma*	0	2
S. stercoralis, *N. americanus* and *A. lumbricoides*	0	2
A. lumbricoides and *Ancylostoma*	0	2†
Negative	71	29

*Analysis of the number of samples detected as single infection showed that two samples had single *A. lumbricoides* by real-time PCR, whereas microscopy showed three samples with only *A. lumbricoides*.

†The one sample not detected by the real-time PCR as single infection was actually detected as multiple infections of *A. lumbricoides* and *Ancylostoma*.

TABLE 3

Real-time polymerase chain reaction (PCR) median cycle threshold (Ct) value in samples that were found positive or negative with microscopy

Organism	Microscopy	Real-time PCR	
		Positive	Median (Ct-value range)
Hookworm	Positive	1	34
	Negative	11 *(Ancylostoma)*	34 (25 < Ct < 36)
	Negative	21 *(Necator americanus)*	34 (28 < Ct < 39)
Ascaris lumbricoides	Positive	3	26 (23 < Ct < 30)
	Negative	3	31 (30 < Ct < 34)
Strongyloides stercoralis	Positive	2	27.5 (24 < Ct < 31)
	Negative	28	36 (24 < Ct < 39)

Six of 77 samples were positive for intestinal parasites by microscopic examination using either direct wet smear, zinc sulphate flotation method, and/or Kato-Katz thick smear and all were confirmed positive by real-time PCR. There were no cases of helminth infection that were detected only with microscopy. *Ancylostoma*-, *N. americanus*-, *A. lumbricoides*-, and *S. stercoralis*-specific DNA amplification was shown in a total of 48 samples. The median Ct values of real-time PCR positive samples are summarized in Table 3. Table 4 shows the larvae and egg counts for positive samples using the traditional parasitological techniques.

The difference in the total detection rate between microscopy and the pentaplex real-time PCR assay was statistically significant ($P \leq 0.05$; χ^2 test) as shown in Table 5, with eight times greater detection by real-time PCR than microscopic examination. There was no significant difference (Fisher's exact test, $P < 0.05$) between the number of samples in which *N. americanus* was detected with real-time PCR (N = 21) and the number of samples positive for *Ancylostoma* (N = 12).

DISCUSSION

Soil-transmitted helminth infections are strongly associated with chronic adverse effects on health and nutritional status of the infected cases.[20] Studies on STH have shown that infections with more than one nematode are much more common than single infections, especially in low-income countries.[21–24] Moreover, the efficacy, dose, and timing of anthelmintic treatment is depending on the helminth species.[1] For example, common treatment of STHs, albendazole and/or mebendazole, shows good efficacy for *Ascaris* and hookworm, moderate

TABLE 4

Egg count for samples positive by real-time polymerase chain reaction (PCR) and parasitological techniques*

ID sample	Organism detected	Parasitological techniques		
		Direct smear (no. of eggs/larvae)	Kato Katz (no. of eggs; EPG)	Zinc sulphate (no. of eggs/larvae)
L28	*Strongyloides stercoralis*	+ (17)	–	–
L32	*Ascaris lumbricoides*	+ (NA)	+ (1584)	+ (NA)
L40	*Hookworm*	+ (3)	–	–
L55	*A. lumbricoides*	+ (NA)	+ (240)	+ (NA)
SH5	*A. lumbricoides*	+ (NA)	+ (144)	+ (NA)
S1	*S. stercoralis*	+ (8)	–	–

* + = positive; – = negative; NA = not available.

TABLE 5

Statistical analysis of the comparison of detection of *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Ancylostoma,* and *Necator americanus* by parasitological techniques and real-time polymerase chain reaction (PCR)

Variable	*N*	Positive frequency (%)	Negative frequency (%)	χ^2 statistic (df)	*P* value
Detection method used					
Microscopy	77	6 (8.0)	71 (92.0)	3.931(1)	< 0.05*
Real-time PCR		48 (62.3)	29 (37.7)		

* Chi-square test.

efficacy for *T. trichiura* but fails to clear *S. stercoralis*.[1,25–27] Furthermore, it has been reported that *A. duodenale* is more easily removed by bephenium, whereas tetrachloroethylene is more efficient for the treatment against *N. americanus*.[28]

The traditional method for the diagnosis of these infections is microscopic examination, using either direct fecal smear or various fecal concentration methods, which are labor-intensive, time-consuming, and not sensitive. For example, the Kato-Katz technique increases the detection rates of some helminth species and provides quantitative results; however, the diagnosis of hookworm is hampered by the deterioration of these eggs in a short period of time, differentiation between hookworm species is not possible, and the detection *S. stercoralis* larvae is poor.[26,29–31] These diagnostic limitations can be avoided with real-time PCR because it allows simultaneous detection and quantification of different targets and has been shown to be highly sensitive and specific for detection of many intestinal parasites.[9,12–15,32–37]

In this study, a pentaplex real-time PCR was evaluated to detect infections with the human roundworm, two species of hookworm and threadworm. An additional fifth primer and probe set was used for the amplification and detection of an internal control for the detection of possible inhibition of the amplification reaction by fecal contaminants. This pentaplex real-time PCR proved to be highly sensitive detecting low DNA copy numbers, i.e., 10 copies for *A. duodenale*, *A. lumbricoides*, and *S. stercoralis*, and 10^3 copies for *N. americanus*.

In 42 of 77 fecal samples in which none of the helminth ova or larvae were detected with microscopy, specific DNA amplification was shown for one or more helminth species. The higher Ct values (i.e., low DNA load) found in the *A. lumbricoides* and *S. stercoralis* microscopy-negative samples as compared with the lower Ct values in the microscopy-positive samples indicates that the high sensitivity of the pentaplex real-time PCR allows the detection of light infections that are missed with microscopic examination. In this study population, such light infections that could be detected with PCR only were found in the majority of cases. These findings are consistent with previous studies in which these real-time PCRs in other multiplex or singleplex formats were evaluated against different microscopic techniques.[9,15,37]

Nevertheless, there are several limitations of this study. Ascertainment of prior anthelminthic use was not made, thus this could potentially have influenced the discrepancy between microscopy and the real-time PCR. Because the PCR-positive results of the above 42 samples were not verified by any other method, the possibility remains that some of these could be false positives. Another limitation of the study was that only symptomatic patients were enrolled, which likely represented infections of higher intensity than that in asymptomatic infected individuals. In this study, fairly low egg counts were found. Studies to compare this novel multiplex PCR with microscopy in settings with different parasite endemicity are planned in the near future.

In comparison with conventional PCR, real-time PCR offers prevention of cross-contamination, efficient multiplexing of multiple targets, and moreover savings in terms of cost and labor time. Cost comparisons in our laboratory showed that each multiplex real-time PCR assay costs approximately US$2.61, whereas conventional PCR, followed by agarose gel electrophoresis cost US$6.43 per assay. With regard to time, multiplex real-time PCR takes about 120 minutes as compared with conventional PCR followed by agarose gel electrophoresis, which required ~210 minutes. The cost of microscopy using three wet smears, and $ZnSO_4$ concentration technique followed by examination of six slides (three from surface sample, and three sediment samples), is approximately US$2.60 per sample, and the time taken is ~170 minutes. Surprisingly, this is similar to the cost of the reagents and disposables to run real-time PCR. However, traditional technique is insensitive and samples need to be examined by an experienced microscopist.

The eggs and larvae of *N. americanus* and *Ancylostoma* species are morphologically similar and difficult to differentiate with microscopy. However, infection by *A. duodenale* causes 2- to 10-fold higher blood losses compared with *N. americanus*.[30] Interestingly in this study both *N. americanus* and *Ancylostoma* species were found in this area of Malaysia. Historically, *N. americanus* has been the primary hookworm species in tropical countries of Southeast Asia including Malaysia, whereas *A. duodenale* has been reported to prefer cooler and drier places.[1,6] According to the NCBI BLAST search, the primers and probe that were designed from the Internal Transcribed Spacer 2 (ITS2) sequence of *A. duodenale* are likely to amplify and detect *Ancylostoma ceylanicum* as well. Further study to identify the *Ancylostoma* species involved will be performed in the near future.

Misdiagnosed, underdiagnosed, or overdiagnosed STH infections often occur when the intensity of the parasites is relatively low and/or the morphology of helminth species are closely related.[38,39] This study shows that the traditional parasitological diagnosis was clearly less sensitive than real-time PCR. In most countries in the world, even in developing nations, there is a trend of a decrease in the number of personnel who are well trained in microscopic identification of parasite ova and larvae. Diagnostic methods that are not dependent on skilled microscopists are needed; therefore, applications of molecular diagnostics are expected to increase. The assay described in this study provides a worthwhile alternative diagnostic method in laboratory diagnosis and epidemiological studies of soil-transmitted helminth infections.

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