



RESEARCH ARTICLE

A conventional multiplex PCR for the detection of four common soil-transmitted nematodes in human feces: development and validation

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ABSTRACT

Soil-transmitted helminth (STH) infections, mainly caused by *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms, are among the most common intestinal parasites that infect humans. The infections are widely distributed throughout tropical and subtropical countries, including Malaysia, particularly in underprivileged communities. Microscopic and culture techniques have been used as a gold standard for diagnostic techniques. However, these methods yield low sensitivity and specificity, laborious and time-consuming. Therefore, simple, rapid, and accurate alternative methods are needed for the simultaneous detection of STH infections. Although advanced technologies such as real-time multiplex PCR have been established, the use of this technique as a routine diagnostic is limited due to the high cost of the instrument. Therefore, a single-round multiplex conventional PCR assay for rapid detection of four STH species in the fecal sample was developed in this study. To perform the single-round multiplex PCR, each pair of species-specific primers was selected from target genes, including *Ancylostoma duodenale* (Internal Transcribed Spacer 2; accession No. AJ001594; 156 base pair), *Necator americanus* (ITS 2; accession No. AJ001599; 225 base pair), *Ascaris lumbricoides* (Internal Transcribed Spacer 1; accession No. AJ000895; 334 base pair) and *Trichuris trichiura* (partial ITS 1, 5.8s rRNA and partial ITS 2; accession No. AM992981; 518 base pair). The results showed that the newly designed primers could detect the DNA of STH at low concentrations (0.001 ng/μl) with no cross-amplification with other species. This assay enables the differentiation of single infections as well as mixed infections. It could be used as an alternative and is a convenient method for the detection of STHs, especially for the differentiation of *N. americanus* and *A. duodenale*.

Keywords: Multiplex PCR; soil-transmitted helminths (STHs); intestinal nematodes; Malaysia.

INTRODUCTION

Soil-transmitted helminth (STH) infections are among the most common infections worldwide that affect the poorest and most deprived communities. More than 1.5 billion people, or 24% of the world's population, are infected with STH infections worldwide (Wardell *et al.*, 2017; WHO, 2018; Han *et al.*, 2019; Kaewpitoon *et al.*, 2019). The main species that infect humans are the roundworm (*Ascaris lumbricoides*), whipworm (*Trichuris trichiura*), and hookworms (*Necator americanus* and *Ancylostoma duodenale*). The World Health Organization (WHO) and Disease Control and Prevention (CDC) have recognized STH infections as one of the most neglected tropical diseases under the same category as other infectious diseases (Hotez, 2009; Wardell *et al.*, 2017). More than a

quarter of the world's population is at risk for infection with the soil-transmitted helminths (STHs). The infections are most prevalent in tropical and subtropical areas, with the most significant numbers occurring in sub-Saharan Africa, the Americas, China, and East Asia (Pullan *et al.*, 2014; WHO, 2018; Darlan *et al.*, 2019). In Malaysia, STH infections are prevalent in the poor and socioeconomically deprived communities (Ngui *et al.*, 2015; Rajoo *et al.*, 2017; Mohd Shaharuddin *et al.*, 2018; Muslim *et al.*, 2019).

Infection with the STHs seldom causes death. Instead, the burden of disease is related less to mortality than to the chronic detrimental effect on the hosts' health and nutritional status (Hotez *et al.*, 2009). Soil-transmitted helminths (STHs) impair the nutritional status of the people they infect in multiple ways. The STHs feed on host tissues,

including blood, which leads to a loss of iron and protein. Hookworms cause chronic intestinal blood loss that can result in iron deficiency anaemia (IDA) and protein malnutrition (Bethony *et al.*, 2006). Because of their underlying poor iron status, children, women of reproductive age, and pregnant women are frequently the ones most susceptible to developing hookworm anaemia (Bethony *et al.*, 2006). Severe IDA due to hookworm infections during pregnancy can have adverse effects on the mother, foetus, and neonates (Bethony *et al.*, 2006). The worms increase the malabsorption of nutrients. *A. lumbricoides* may compete for vitamin A in the intestine. *A. lumbricoides* can also cause intestinal obstruction among children (Andrade *et al.*, 2015). Severe infection of *T. trichiura* leads to *Trichuris* dysentery syndrome (TDS), characterized by chronic dysentery, rectal prolapse, vitamin A deficiency, anaemia, and growth retardation, particularly in children (Khuroo *et al.*, 2010).

The World Health Organization (WHO) has recognized STH infections as one of the most important causes of physical and intellectual impairment among school-aged children (Bethony *et al.*, 2006). Nonetheless, despite their significant public health importance, they remain largely neglected by the medical and international community. This neglect occurs due to several factors such as the most affected people belonging to the poorest and disadvantaged communities who often live in remote, rural areas, urban slums, or in conflict zones (Hotez *et al.*, 2009). With little political voice in these disadvantaged communities, STH infections have a low profile and status in public health priorities. They are not highly visible and do not cause explosive outbreaks that attract public and media attention (Hotez *et al.*, 2009).

Most research conducted on the epidemiology of STHs has relied on the use of conventional microscopy for the identification of eggs in feces and third-stage larvae (L3) through the coproculture technique. This method is technically simple and economical. Nevertheless, this technique has low sensitivity, especially in cases of mild infection. Moreover, some nematode eggs such as hookworm are morphologically indistinguishable (Al-Kubaisy *et al.*, 2014; O'Connell & Nutman, 2016; Mbong Ngwese *et al.*, 2020). Alternatively, several diagnostic techniques have been developed to identify and differentiate STH infection, particularly DNA-based techniques. The advent of these molecular methods has provided more specific and sensitive techniques for diagnosing STH infections compared to traditional microscopy techniques (Llewellyn *et al.*, 2016). These approaches have proved to be highly sensitive and specific for the differentiation of STH species. However, there are some limitations, such as the time-consuming amplification process due to the inability of the primer to amplify more than one target region in a single reaction (Areekul *et al.*, 2010; Al-Talib *et al.*, 2019).

This is a strong reason for the use of multiplex PCR. A real-time multiplex PCR using fluorescent detection probes to detect different STH species into one reaction has also been successfully developed (Verweij *et al.*, 2007; Basuni *et al.*, 2012; Stracke *et al.*, 2019). In addition to that, another molecular technique based on real-time PCR coupled with High Resolution melting (HRM) analysis has been developed for rapid detection and speciation of hookworm species in a human sample (Ngui *et al.*, 2012). Although the techniques are beneficial and practical, the high cost and need for specialized instruments such as real-time PCR machines limit routine diagnosis applications and are unlikely to be available in a standard laboratory.

To date, several multiplex assays based on conventional PCR have been developed to simultaneously detect STHs (Phuphisut *et al.*, 2014; Sanprasert *et al.*, 2019). However, Phuphisut *et al.* only targeted three species, namely *A. lumbricoides*, *T. trichiura*, *N. americanus* (Phuphisut *et al.*, 2014). Likewise, Sanprasert *et al.* also targeted three species, which are *A. lumbricoides*, *N. americanus*, and *S. stercoralis* (Sanprasert *et al.*, 2019). In this study, a multiplex assay based on conventional PCR was developed and evaluated to simultaneously detect four major STHs, namely *A. lumbricoides*, *T. trichiura*, *N. americanus*, and *A. duodenale* in a human fecal sample. The results were compared with the standard monoplex PCR and microscopy technique in a field-collected sample.

MATERIALS AND METHODS

Control samples preparation

Well-defined positive controls of *A. lumbricoides*, *T. trichiura*, *N. americanus*, *A. duodenale*, and *A. lumbricoides* were obtained from the unused and archived specimens from our previous studies (Rajoo *et al.*, 2017; Mohd-Shaharuddin *et al.*, 2018; Hassan *et al.*, 2021). These samples were collected from the indigenous communities. Some of the archived specimens were acquired from the Parasite Southeast Asian Diagnostic (Para:SEAD) Laboratory at the Department of Parasitology, Faculty of Medicine, Universiti Malaya. These samples were sent to the Para:SEAD laboratory to diagnose parasitic infections from a patient presenting with gastrointestinal symptoms attending the Universiti Malaya Medical Centre (UMMC). These samples were examined with formalin ether concentration technique followed by microscopy examination. The microscopy-positive samples were then subjected to PCR and sequenced for species confirmation, especially hookworm species (Rajoo *et al.*, 2017; Mohd-Shaharuddin *et al.*, 2018; Hassan *et al.*, 2021). In addition to that, control specimens for *Ancylostoma ceylanicum*, *Trichuris vulpis*, *Ascaris suum*, *Toxocara canis*, *Toxocara cati*, *Taenia* spp., *Entamoeba* spp., *Cryptosporidium* spp., *Microsporidia* spp., *Giardia* spp., and *Blastocystis* spp. were obtained from our archived specimens. The University of Malaya Medical Centre Ethics Committee approved this study (IRB Ref. No.655.17).

Genomic DNA preparation

Genomic DNA was extracted using the FavorPrep™ Stool DNA Isolation Mini Kit (Favorgen Biotech Corp, cat. no. FASTI 001-1, Taiwan) according to the manufacturer's instructions. Briefly, approximately 0.2 to 0.3 g of stool pellet was added into the PowerBead Tube and incubated at 70°C for 10 minutes with the cell lysis and disruption agent provided in the kit. The stool samples were then subjected to homogenization and lysis procedures for complete cell lysis by mechanical shaking (vortexing) using VX-200 Vortex Mixer (Labnet International Inc., NJ, USA).

The adult worms were washed with sterile normal saline and chopped on ice with a sterile scalpel blade. The chopped worm's tissue fragment was homogenized in a pestle homogenizer and extracted using the FavorPrep™ Tissue DNA Extraction Mini Kit (Favorgen Biotech Corp, cat. no. FASTI 001-1, Taiwan), according to the manufacturer's instructions. Briefly, homogenated worms were resuspended in lysis buffer and proteinase K and incubated at 60°C for 1 to 3 hours. Next, the homogenated worms underwent lysis procedures for complete lysis using the same vortex mixer for stool samples extraction. A final elution of the DNA was made in 100 µl. The DNA concentration and purity were

qualitatively determined using a NanoPhotometer (IMPLEN, Germany). The extracted DNA was stored at -20°C until further use.

Primer design of the multiplex PCR

To perform multiplex PCR assay, each pair of species-specific primers were designed to specifically amplify the target region of *A. lumbricoides*, *T. trichiura*, *N. americanus*, and *A. duodenale* from previously published sequences (GenBank accession numbers AJ000895.1, AM992981.1, AJ001599.1, and AJ001594.1). All published sequences of each STH species were aligned and edited to obtain the consensus sequence using the BioEdit Sequence Alignment version 7.0.9 program (Hall, 1999) and CLUSTAL-W analysis (Thompson et al., 1994). A single pair for each species was designed separately with the aid of sequence analysis and Primer Express software (Applied Biosystems, Inc., CA, USA) followed by *in silico* PCR analysis (<http://insilico.ehu.es/PCR/>). This ensured that the designed primers targeted the region of interest before forming the desired degenerate primers. In addition to that, to ensure the designed primers targeted the genomic region of interest, the primers were cross-checked using the Basic Local Alignment Search Tool (BLAST) by National Centre for Biotechnology Information (NCBI) (Bethesda, MD, USA). The primer sequences for the four genes and the expected PCR product sizes are shown in Table 1 and Supplementary Data.

Preliminary optimization of the primer

For preliminary optimization of the primers, gradient PCR using conventional PCR was conducted using a wide range of well-defined STH DNA to obtain the optimal annealing temperature. Briefly, the singleplex PCR of each STH species was performed using 25 µl of a PCR mixture containing 0.5 U/µl of master mix (Bioline USA Inc. USA), 10 picomoles of each forward and reverse primer, and 2 µl of the DNA template. The mixture was heated at 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds (denaturing), 50°C to 60°C for 30 seconds (gradient annealing temperature), 72°C for 30 seconds (extension), and a final extension at 72°C for 7 minutes. DNA blank (DNase/RNase free water, Sigma Cat. no. W4502, St. Louis, MO) and positive genomic DNA were also included in each PCR optimization. The PCR cycle was performed in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The gel was stained with SYBR® Safe DNA gel stain (Invitrogen, Cergy Pontoise, France) to visualize the DNA fragments under an ultraviolet (UV) transilluminator.

The positive amplicons were then purified using the QIAquick Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, except that the final

elution of DNA was 30 µl of elution buffer instead of 50 µl. The purified PCR products were then sent for sequencing to confirm that the amplified PCR product was from the targeted species. The sequence chromatograms were viewed using Sequence Scanner (Applied Biosystems, Inc., CA, USA). Both forward and reverse sequences were manually aligned, and the consensus sequence was created using the BioEdit version 7.0.9 (Hall, 1999). Homology searches were carried out using the Basic Local Alignment Search Tool (BLAST) by National Centre for Biotechnology Information (NCBI) reference sequences.

Cloning and DNA sequencing of the target region

The positive amplicons for each STH species from singleplex PCR were then cloned using pEASY®-T5 cloning kit (Transgen Biotech, Beijing, China). The isolated DNA fragment (often referred to as inserts) was obtained from the product of the previous singleplex PCR. To isolate the DNA fragment, PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Germany). Then, the DNA fragment was ligated into the pEASY®-T5 Zero Cloning Vector. The ligated products were added to Trans-T1 competent *Escherichia coli* cells during the transformation step. Since pEASY®-T5 contains a suicide gene, the expression of the gene was disrupted during the ligation step. Cells that contained the non-recombinant vector were killed upon plating. Therefore, blue/white screening was not required for the selection and identification of recombinant clones. The selected recombinant clones were sent for sequencing to verify the presence of the target sequence. The correct inserted recombinant plasmid DNA was purified using EasyPure® Plasmid Miniprep Kit (Transgen, Beijing, China) according to the manufacturer's instructions. A 10-fold serial dilution of the recombinant plasmid DNA was used to develop and optimize the multiplex assay.

Development and optimization of the multiplex PCR assay

The multiplex PCR amplification reaction was performed in a 25 µl reaction mixture containing 0.5 U/µl master mix (Bioline USA Inc. USA), 5 picomoles of each forward and reverse primer for every STH species, 1 µl of positive control from each DNA template, and 3 µl of DNA blank (DNase/RNase free water, Sigma Cat. no. W4502, St. Louis, MO). A negative control (reagent mixture without template DNA) was included in each reaction. The amplification was set at 94°C for 5 minutes (initial heating) followed by 30 cycles of 94°C for 30 seconds (denaturing), 55°C for 30 seconds (optimal annealing temperature), 72°C for 30 seconds (extension), and a final extension at 72°C for 7 minutes. The amplification was performed in a MyCycler Thermal Cycler (Bio-Rad

Table 1. Oligonucleotide sequences and targeted base pair for each of the STH species

Species	Accession no.	Sequences	Base pair (bp)
<i>Ancylostoma duodenale</i>	AJ001594.1 Internal Transcribed Spacer 2 (ITS 2)	<i>Ancylostoma</i> -F: ACTGTTTGTGCAACGGCACT <i>Ancylostoma</i> -R: GCCGAAACGTTCTAAAGTCG	156
<i>Necator americanus</i>	AJ001599.1 Internal Transcribed Spacer 2 (ITS 2)	<i>Necator</i> -F: GTGTTTCAGCAATTCCTGTTT <i>Necator</i> -R: TTGCAATGACACATCCACA	225
<i>Ascaris lumbricoides</i>	AJ000895.1 Internal Transcribed Spacer 1 (ITS 1)	<i>Ascaris</i> -F: GTCTCCGAACGTGCACATAA <i>Ascaris</i> -R: CTCCAAGCTGAGGCTCATTG	334
<i>Trichuris trichiura</i>	AM992981.1 ITS 1 (partial), 5.8S rRNA gene and ITS 2 (partial)	<i>Trichuris</i> -F: AGGCAGCAGCAATTTTCACT <i>Trichuris</i> -R: CGTAGCGTCGACATCTTTGA	518

Laboratories, Hercules, CA, USA). The PCR products were electrophoresed using 2% agarose gel containing SYBR® Safe DNA gel stain (Invitrogen, Cergy Pontoise, France). The agarose gel was viewed under the ultraviolet (UV) transilluminator. To confirm that the amplified PCR product was specific to the targeted species, each of the amplified PCR bands was cut from the agarose gel with sterile and disposable scalpel blades. The cut agarose gel was purified and sent for sequencing. The sequence was analyzed similarly as described above.

Sensitivity, specificity analysis, and random blind screening

Analytical sensitivity was evaluated using 10-fold serial dilutions of the positive control genomic DNA ranging from 10^{-1} to 10^{-5} to detect the lowest detectable DNA concentration in the multiplex PCR. The sensitivity and specificity of the conventional multiplex PCR system to detect STH species were compared to a conventional singleplex PCR as the molecular gold standard. The microscopy examination results were used as a reference for the presence of STH species.

The specificity was tested using a wide range of genomic DNA of various intestinal parasites that were endemic and commonly reported in Malaysia, including *Ancylostoma ceylanicum*, *Trichuris vulpis*, *Ascaris suum*, *Toxocara canis*, *Toxocara cati*, *Entamoeba* spp., *Cryptosporidium* spp., *Microsporidia* spp., *Giardia* spp., and *Blastocystis* spp. In addition to that, the assay was also tested using DNA isolated from an individual with no history of intestinal parasitic infections. To validate the sensitivity and specificity of the multiplex conventional PCR assay for species determination, a random blind 100 DNA samples from a previously field-collected survey were carried out for comparison purposes.

RESULTS

Singleplex and multiplex PCR assay

Alignment of the gene sequence from four STH species of *A. lumbricoides*, *T. trichiura*, *N. americanus*, and *A. duodenale* was performed using the CLUSTAL W program (Thompson et al., 1994) and BioEdit version 7.0.9 (Hall, 1999) to obtain the consensus sequences for primer design. The selected primers were tested through a BLAST search and *in silico* PCR.

The PCR conditions for each primer pair were optimized and successfully amplified the expected genes of interest as confirmed by agarose gel electrophoresis and sequencing [~156 bp for *A. duodenale*, ~225 bp for *N. americanus*, ~334 bp for *A. lumbricoides*, and ~518 bp for *T. trichiura* (Figure 1A-1D, respectively)].

The optimized primer pairs were then mixed to perform a multiplex PCR to detect the four STH species. In the multiplex PCR reaction, all four different-sized amplicons were simultaneously amplified (Figure 2A-2B). The PCR products of each STH species were cloned using pEASY®- T5 cloning kit (Transgen Biotech, Beijing, China) and used as the 'plasmid controls' in the subsequent experiments.

Sensitivity and specificity analysis

The sensitivity of the multiplex PCR assay was assessed using 10-fold serial dilutions for each individual STHs ranging from 10^{-1} to 10^{-5} to determine the lowest detectable DNA concentration. The final detection limit of *A. lumbricoides* was 10^{-4} (0.001 ng/ μ l) followed by *N. americanus* 10^{-4} (0.001 ng/ μ l), *A. duodenale* 10^{-3} (0.01 ng/ μ l), and *T. trichiura* 10^{-2} (0.1 ng/ μ l). Subsequently, a total of 10 possible combinations of cloned DNA mixture to create two and three-species-simulated mixed infections with equal DNA clone concentration (100 pg/ μ l) was performed, as shown in Figure 3.

Species-specific primers of *A. lumbricoides*, *N. americanus*, *A. duodenale*, and *T. trichiura* were used to evaluate the specificity of the multiplex conventional PCR with quadruple plasmid controls. In general, the analytical specificity of the assay to identify the STH species was 100% accurate. No cross-amplifications were recorded for each primer set and plasmid DNA. In addition to that, there was also no cross-amplification reported with the DNA template of other intestinal parasites (*Ancylostoma ceylanicum*, *Trichuris vulpis*, *Ascaris suum*, *Toxocara canis*, *Toxocara cati*, *Taenia* spp., *Entamoeba* spp., *Cryptosporidium* spp., *Microsporidia* spp., *Giardia* spp., and *Blastocystis* spp.).

Random blind screening of the field-collected sample

To validate the performance of the multiplex conventional PCR assay, a total of 100 stool samples were examined using microscopy and multiplex PCR assay. Based on the microscopy

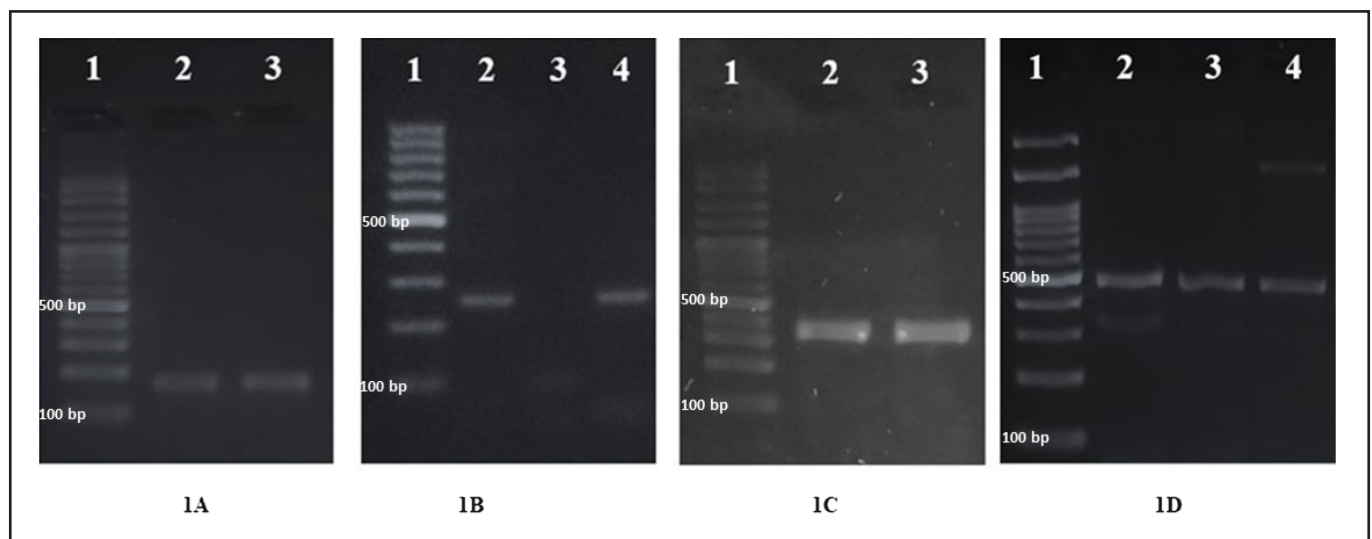


Figure 1. Singleplex PCR assay results. A representative electrophoresis gel result (2%) of PCR products of *A. duodenale*, *N. americanus*, *A. lumbricoides*, and *T. trichiura*. Figure 1A, *A. duodenale* targeted band (156-bp); 1B, *N. americanus* targeted band (225-bp); 1C, *A. lumbricoides* targeted band (334-bp); 1D, *T. trichiura* targeted band (518-bp). Lane 1= DNA ladder 100-base pairs (GeneDireX, Inc).

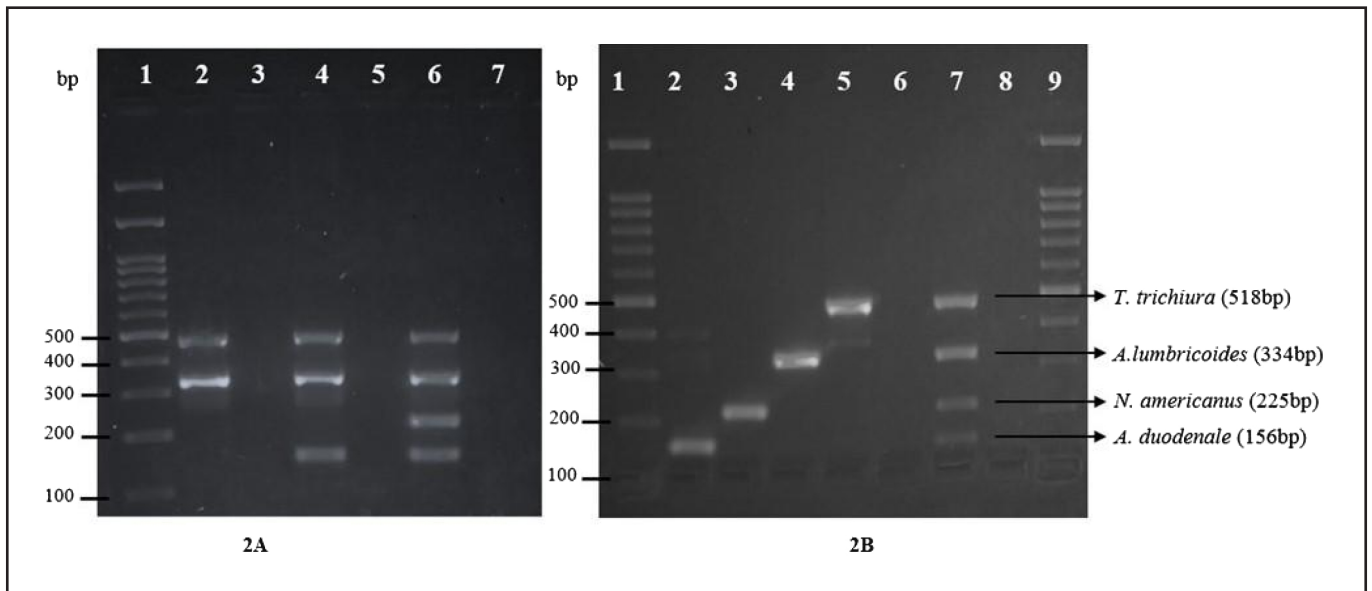


Figure 2. Singleplex and multiplex PCR assay results. Electrophoresis gel result (2%) of PCR products of *A. duodenale*, *N. americanus*, *A. lumbricoides*, and *T. trichiura*. Figure 2A, lane 2, *T. trichiura* and *A. lumbricoides*; lane 4, *T. trichiura*, *A. lumbricoides* and *A. duodenale*; lane 6, *T. trichiura*, *A. lumbricoides*, *N. americanus* and *A. duodenale*; lane 3, 5, 7, DNA blank. Figure 2B, lane 2, *A. duodenale*; lane 3, *N. americanus*; lane 4, *A. lumbricoides*; lane 5, *T. trichiura*; lane 7, multiplex PCR ladder containing four STH species; lane 6, DNA blank. Lane 1 and 9= DNA ladder 100-base pairs (GeneDireX, Inc, Taiwan).

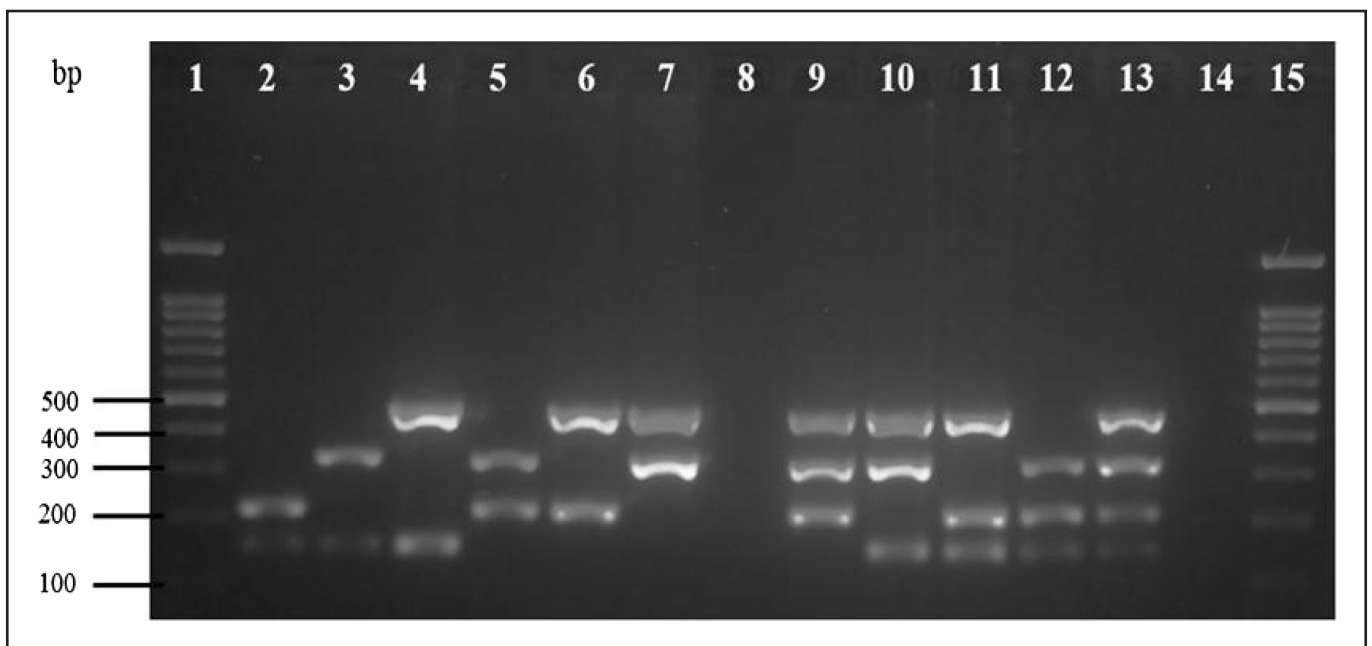


Figure 3. Multiplex conventional PCR assay results based on simulated mixed clone DNA. Lane 2, *A. duodenale* and *N. americanus*; lane 3, *A. duodenale* and *A. lumbricoides*; lane 4, *A. duodenale* and *T. trichiura*; lane 5, *N. americanus* and *A. lumbricoides*; lane 6, *N. americanus* and *T. trichiura*; lane 7, *A. lumbricoides* and *T. trichiura*; lane 9, *T. trichiura*, *A. lumbricoides* and *N. americanus*; lane 10, *T. trichiura*, *A. lumbricoides* and *A. duodenale*; lane 11, *T. trichiura*, *N. americanus* and *A. duodenale*; lane 12, *A. lumbricoides*, *N. americanus* and *A. duodenale*; lane 13, multiplex PCR ladder containing four STH species; lane 8 and 14, DNA blank. Lanes 1 and 15, 100-bp DNA ladder (GeneDireX, Inc, Taiwan).

examination, 60% (60/100) of the stool samples were reported positive with at least one STH species. In contrast, the multiplex PCR assay showed that 88% (88/100) of the samples were reported positive with STH infections. A detailed comparison of multiplex PCR and microscopy/monoplex PCR for detection of STHs is presented in Table 2. The sensitivity and specificity of the multiplex PCR assay and microscopy

method were also evaluated in this study. Both methods reported 100% specificity (the ability of the assay to identify actual positive infections), and with regards to the sensitivity (the ability of the assay to identify true negative infections), the multiplex conventional PCR assay (100%) reported higher sensitivity (the ability of the assay to identify true negative infections) compared to the microscopy method (71.6%).

Table 2. Comparison of multiplex PCR assay and microscopy in the detection of STH infections (N=100)

Multiplex PCR	Positive	Microscopy/ monoplex PCR*	
		Positive	Negative
Positive			
Single Infection			
<i>A. lumbricoides</i>	20	10	10
<i>T. trichiura</i>	18	10	8
<i>N. americanus</i>	5	5	0
<i>A. duodenale</i>	3	3	0
Double Infections			
<i>A. lumbricoides</i> + <i>T. trichiura</i>	17	7	10
<i>A. lumbricoides</i> + <i>N. americanus</i>	5	5	0
<i>A. lumbricoides</i> + <i>A. duodenale</i>	5	5	0
<i>T. trichiura</i> + <i>A. duodenale</i>	5	5	0
<i>T. trichiura</i> + <i>N. americanus</i>	5	5	0
Triple Infections			
<i>A. lumbricoides</i> + <i>T. trichiura</i> + <i>N. americanus</i>	5	5	0
Total#	88	60	28

#12 samples were detected negative for any STH infections by both methods.

*Details have been published elsewhere (Rajoo et al., 2017; Mohd-Shaharuddin et al., 2018; Hassan et al., 2021).

DISCUSSION

STH infections in the areas of high endemicity have an enormous socio-economic and developmental impact on the infected populations. Global strategies have been implemented through the WHO and various non-governmental organization (NGO) partnerships to reduce the burden of STH infections through mass drug administration and morbidity control. However, a more practical approach would be integrating laboratory testing using a highly sensitive and specific, rapid, accurate, and economically efficient diagnostic tool as an adjunct of treatment and to control management effectiveness (Sanprasert et al., 2019). Co-infection of STH with more than one species is common in an infected person. The efficacy, dose, and timing of anthelmintic treatment depend on the helminth species, although the mild symptoms of STH are similar. Therefore, a simultaneous diagnosis of STH infections is required.

To date, microscopic examination such as direct smear, Kato-Katz, formalin-ether concentration technique (FETC), and floatation technique to diagnose helminth eggs or larva in fecal samples have been used as the standard method to diagnose STH infections. Although this technique is economical and straightforward, this technique possesses low sensitivity, especially in cases with a mild infection. One alternative is a molecular-based technique, which is a highly sensitive technique with high throughput capacity that can be used for STH diagnosis. Different protocols have been developed for PCR assays based on single, nested, and multiplex using conventional and real-time PCR machines (Verweij et al., 2007; Areekul et al., 2010; Basuni et al., 2011; Ngui et al., 2012; Phuphisut et al., 2014; Al-Talib et al., 2019; Deng et al., 2019; Sanprasert et al., 2019; Stracke et al., 2019). Depending on the target of interest and the available resources, the choice of the PCR varies from a simple semi-

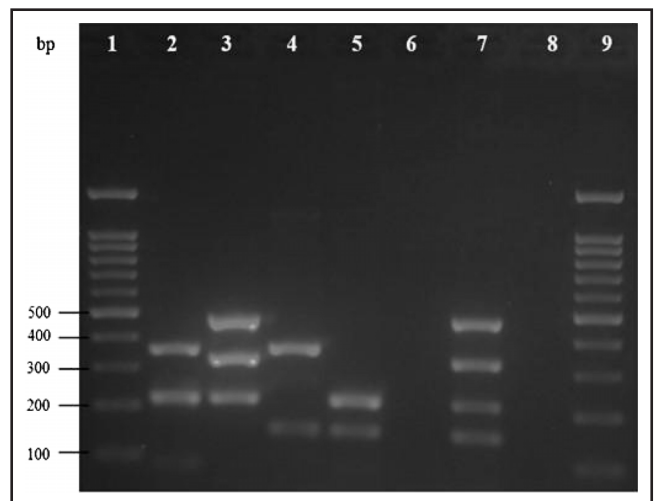


Figure 4. Multiplex conventional PCR assay results based on the simulated random blind screening samples. Lanes 2 to 5, random mixed infections of double and triple infections of four STH species; lane 2, positive samples *A. lumbricoides* and *N. americanus*; lane 3, positive samples *T. trichiura*, *A. lumbricoides* and *N. americanus*; lane 4, positive samples *A. lumbricoides* and *A. duodenale*; lane 5, positive samples *N. americanus* and *A. duodenale*. Lane 6, human healthy control DNA; lane 7, multiplex PCR containing four STH species; lane 8, DNA blank; lanes 1 and 9, 100-bp DNA ladder (GeneDireX, Inc, Taiwan).

quantitative to quantitative real-time PCR. These techniques have been shown in many cases to have improved sensitivity and specificity compared to microscopy evaluations. Multiplex PCR is one of the research priorities for STH infections diagnosis in the future. Generally, the drawbacks of the multiplex PCR system are the difficulties in designing the primers and the cumbersome optimization steps needed to establish a highly sensitive and specific assay, while avoiding cross-infection between the species.

This study has successfully developed and evaluated a conventional multiplex PCR for synchronous detection of four important intestinal nematode species (*T. trichiura*, *A. lumbricoides*, *N. americanus*, and *A. duodenale*). Using a pair of species-specific primers, amplified fragments of different sizes were obtained. Overall, the optimization steps and sensitivity and specificity tests were performed using four STH species from stool and adult worm samples. The sensitivity and specificity of the assay were also ensured by a random blind screening (n=100) (Figure 4) as well as experimental mixed infections from cloned DNA (Figure 3). The results were aligned with previous outcomes. In addition to that, the multiplex conventional PCR assay had a high sensitivity, with the final detection limit as low as 0.001 ng/ μ of DNA template for *A. lumbricoides* followed by *N. americanus* (0.001 ng/ μ l), *A. duodenale* (0.01 ng/ μ l), and *T. trichiura* (0.1 ng/ μ l). No cross-reactivities between each species-specific primer or other parasites' genomic DNA, including *Entamoeba* spp., *Cryptosporidium* spp., Microsporidia spp., *Giardia* spp., *Blastocystis* spp., *Ancylostoma ceylanicum*, *Trichuris vulpis*, *Ascaris suum*, *Toxocara canis*, *Toxocara cati*, and *Taenia* spp. were detected. However, cross-reactivity with other human intestinal parasites should be further investigated. In this study, the specificity and sensitivity for STH identification using the current methods were higher than the previous method.

In mixed infections, the effect of primers and competing DNA can result in inconsistent results in the multiplex PCR assay. This problem was not detected in the PCR assay developed and described in this study. However, the only concern in the evaluation of the conventional multiplex PCR in this study is the lack of naturally acquired mixed infections. The mixture of two or three parasite species using the clones DNA with different DNA ratios used in this study successfully identified the species in all samples. This confirms that the assay in this study is robust and is capable of detecting mixed infections up to a two-species level without any diagnostic constraints.

The multiplex conventional PCR assay is a reliable tool for STH species detection and differentiation. It had a 100% sensitivity and specificity for detecting *T. trichiura*, *A. lumbricoides*, *N. americanus*, and *A. duodenale* (previously confirmed with DNA sequencing). All negative microscopy samples were subjected to multiplex PCR assay to avoid misdiagnosis. None of the samples devoid of detection of STH using microscopy technique was amplified using multiplex assay. In Malaysia, the most common hookworm species reported are *N. americanus* and *A. ceylanicum* (Ngui et al., 2012). In the southern part of Thailand, a low prevalence of *A. duodenale* has been reported in humans compared to *N. americanus* (Anantapruthi et al., 2002). A study performed in a population living in central Thailand and Iran indicated that more than 90% of the population were infected with *N. americanus* than *A. duodenale* (Jiraanankul et al., 2011; Sharifdini et al., 2017). Interestingly, the detection of *A. duodenale* was seen in this study.

The multiplex conventional PCR assay has some advantages compared to other molecular-based assays that use a conventional PCR machine. The multiplex PCR assay is less labor-intensive and faster (reduced hands-on time) due to having only a one-step PCR in a single reaction. This is in contrast with at least two and more steps for the nested and real-time PCR assays. The fewer steps also reduce PCR reagents and consumables (Chew et al., 2012; Hawash et al., 2016; Al-Talib et al., 2019). The time required for this single step multiplex conventional PCR is only 2 hours versus approximately 8-10 hours for nested PCR. In addition to that, the multiplex assay uses a single-tube reaction, and this reduces the risk of carryover and possible external contamination during the transfer of PCR amplicons from primary to secondary PCR mixtures and agarose gel electrophoresis (Chew et al., 2012; Al-Talib et al., 2019; Sanprasert et al., 2019). The multiplex assay is based on the conventional PCR machine, therefore, it is an ideal and affordable method, especially in developing countries. Compared to the real-time PCR machine, the conventional PCR thermal cycler and agarose gel electrophoresis systems are readily available in most laboratories (Momčilović et al., 2019). The multiplex PCR assays also allow large-scale screening of samples within a short period, especially in endemic regions where there are limited resources and experienced microscopists (Chew et al., 2012; Momčilović et al., 2019). The workflow for the microscopic examination can be laborious and time-consuming when dealing with substantial sample sizes.

We acknowledge several shortcomings, and the results obtained should be interpreted with caution. The results of this study relied on crude genomic DNA extracted directly from a fecal sample. The presence of PCR inhibitors, which comprise all substances that have a negative effect on the PCR, is a major drawback of the PCR, which may result in decreased sensitivity or false-negative results. However,

additional steps during the DNA extraction process were performed to reduce fecal inhibitors. This includes extra washing steps to a total of 3 times followed by centrifugation at 4,000 x g for an additional 3 minutes to dry the SDE Mini Column, as recommended by the manufacturer. In addition to that, the choice of commercially available kits for nucleic acid preparation was also taken into consideration in this study. From our experience with various commercially available stool DNA extraction kits, the DNA extraction kit utilized in this study has good reliability and sensitivity. Previous studies have shown that treatment with proteinase K and heat may remove inhibitors from samples more efficiently than other treatment methods (Bergallo et al., 2006; Schrader et al., 2012), which are part of the DNA extraction steps used in this study. Nonetheless, the use of internal/external controls and spike tests could be performed to confirm the absence of PCR inhibitors in the fecal samples in future studies.

In conclusion, the results showed that the conventional PCR machine's multiplex assay is a simple, rapid, sensitive, and specific method for the simultaneous detection of four major STH species in Malaysia. It has the potential as an alternative method to be used in a routine diagnosis of fecal samples in the future.

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Conflict of interest

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY DATA

<https://msptm.org/files/Vol39No1/tb-39-1-016-Hassan-N-A-supplementary-data.pdf>