# TRIPLEX POLYMERASE CHAIN REACTION ASSAY FOR DETECTION OF MAJOR SOIL-TRANSMITTED HELMINTHS, *ASCARIS LUMBRICOIDES*, *TRICHURIS TRICHIURA*, *NECATOR AMERICANUS*, IN FECAL SAMPLES

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**Abstract**. *Ascaris lumbricoides*, *Trichuris trichiura*, and *Necator americanus* are medically important soil-transmitted helminths (STHs) occurring frequently worldwide including Thailand. Fecal examination using a microscope has been recommended as the gold standard for diagnosis of STH infections, but suffers from low sensitivity. Recently, highly sensitive and specific assays, such as multiplex quantitative PCR, has been established, but the high cost and need for special instruments are still barriers limiting their applications in routine diagnosis. Therefore, a conventional multiplex PCR assay, with its lower cost and greater simplicity, was developed, for the simultaneous detection of STHs in fecal samples. The multiplex PCR assay was species-specific to the three STHs, and could detect one copy of DNA target. Compared with microscopic examination of fecal samples, sensitivity and specificity of the multiplex PCR was 87% and 83%, respectively. This multiplex PCR assay provides an alternative method for routine diagnosis of STHs infection, and might be applied for epidemiological studies of STHs in endemic areas.

**Keywords:** soil-transmitted helminth, multiplex PCR, sensitivity, specificity

## INTRODUCTION

Soil-transmitted helminth (STH) infections are one of the most widely studied neglected tropical diseases (NTD), affecting more than two billion people worldwide (WHO, 2011). The disease frequently occurs in remote and poverty-stricken areas where poor sanitation and low education persist (de Silva *et al*, 2003). STH infections are caused mainly by *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms (*Necator americanus* and *Ancylostoma duodenale*) (Hotez *et al*, 2008; Mascarini-Serra, 2011).

*A. lumbricoides* or giant roundworm has a prevalence of approximately 807-1,221 millions worldwide (Bethony *et al*, 2006). Disease symptoms can vary from asymptomatic to severe manifestations, depending on parasite intensity and migration (Khuroo, 1996). In terms of diagnosing the parasites, fecal examination using direct smear or concentration tech-

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niques can readily detect *A. lumbricoides* ova, because the adult female produces as many as 200,000 eggs per day (Murray *et al*, 2005). However, previous reports have suggested egg production is reduced after treatment with anthelminthic drugs which might give rise to false negatives when using microscopic examination (Roberts and Janovy, 2009).

*T. trichiura* or whipworm is another common STH found worldwide. Recent studies suggest infection of the world's population has reached 604-795 millions (Bethony *et al*, 2006). Ineffective treatment with anthelminthic drugs is an important issue (Vercruysse *et al*, 2011). Albendazole has shown low curability, but has an enormous effect in terms of reduced egg production (Bennet and Guyatt, 2000; Vercruysse *et al*, 2011), which might decrease sensitivity of microscopic examination. Therefore, detection using techniques with high sensitivity and specificity, such as polymerase chain reaction (PCR), could offer a better alternative.

*N. americanus* and *A. duodenale* are human hookworms that have been distributed coincidently across all continents, with prevalence of approximately 576-740 millions (Bethony *et al*, 2006). Hookworm infection can cause morbidity with chronic blood loss and anemia, which affects the physical and cognitive growth of children (Hotez *et al*, 2004, 2005). For diagnosis of hookworm, culture technique is an effective method with high sensitivity and specificity (Inés *et al*, 2011), but it is time-consuming and requires experience in larval identification, two significant drawbacks in practical terms. Recently, PCR-based assays have been developed that elicit high sensitivity, specificity and rapidity in detecting hookworm in fecal samples (Verweij *et al*, 2007).

Although conventional methods, such as simple smears, Kato-Katz technique, and the above mentioned culture techniques, are effective means of detecting STH infections in fecal samples, they have limitations in such situations as in light infections and post-anthelminthic drug treatment. As a consequence, PCR is now being commonly used for detection of STHs in fecal samples due to its high sensitivity, specificity and rapidity (Verweij *et al*, 2001; Gruijter *et al*, 2005; Leles *et al*, 2009; Areekul *et al*, 2010). With regards to multiple STH infections, recently, multiplex quantitative PCR has been developed to detect simultaneously intestinal parasites, including STHs (Verweij *et al*, 2007; Basuni *et al*, 2011; Taniuchi *et al*, 2011). However, its high cost, as well as the need for advanced instrument is common criticisms. In order to obtain a simple, rapid, and cost-effective diagnostic tool, multiplex PCR has been developed and successfully detected tapeworms (Yamasaki *et al*, 2004), and for large and small liver fluke (Magalhães *et al*, 2004; Le *et al*, 2006).

In this study, we developed a multiplex PCR assay, which can simultaneously detect STH infections, including *A. lumbricoides*, *T. trichiura*, and *N. americanus*. The assay was evaluated by detecting STH egg DNA in field-collected samples.

## MATERIALS AND METHODS

### Samples

One hundred and fifty-six human fecal samples were collected from three distinct regions, Nakhon Si Thammarat, Samut Sakhon, and Tak provinces, Thailand during 2011-2012. All samples were examined by a modified cellophane thick smear (Kato-Katz) technique (Kato and

Miura, 1954), which presented single or multi-STH infections. Approximately 2 g of each sample were preserved in 5 ml of 70% ethanol.

Adult parasitic helminths, including *A. lumbricoides*, *T. trichiura*, and *N. americanus*, were provided by the Sample Collecting Unit, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and used as control specimens. DNA from *Ascaris suum*, *Schistosoma mekongi*, *Strongyloides stercoralis*, *Taenia* spp, *Enterobius vermicularis*, *Opisthorchis viverrini*, *Haplorchis pumilio*, *Neospora caninum, Cryptosporidium* spp, *Entamoeba histolytica*, and *Giardia lamblia* were used in the determination of specificity of the multiplex PCR assay.

All samples used in this study were obtained from unused specimens and the study was approved by Mahidol University Ethical Review Committee (MUTM 2012-063-01).

**Multiplex PCR**

Adult *A. lumbricoides*, *T. trichiura*, and *N. americanus* were ground in the homogenization buffer (1 M Tris-HCl, 0.5 M EDTA, 10% SDS and 5 M NaCl) and genomic DNA was extracted using FavorPrep™ Tissue Genomic DNA Extraction Mini-Kit (Favorgen, Ping-Tung, Taiwan), according to the manufacturer's instructions. For isolation of fecal DNA, 0.5 ml aliquots of ethanol-preserved fecal samples were washed twice with sterile, double-distilled water ($ddH_2O$), frozen rapidly in liquid nitrogen, homogenized and fecal DNA was extracted using FavorPrep™ Stool DNA Isolation Mini-Kit (Favorgen, Ping-Tung, Taiwan). DNA concentrations were determined using NanoDrop® spectrophotometer ND-1000 (NanoDrop Technologies, Wilington, DE) and samples were stored at -20ºC until analyzed.

Specific primers for detection of *A. lumbricoides*, *T. trichiura*, and *N. americanus* were designed using Primer3 (v.0.4.0) software (Rozen and Skaletsky, 2000) from DNA regions of COI (GenBank Accession No.: EU582499), 18S rDNA (GenBank Accession No.: GQ352548), and ITS1 (GenBank Accession No.: AJ001680), respectively. Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH, Accession No.: XM005253678) was used as internal control for determination of quality of extracted DNA. Primer sequences and amplicon sizes are shown in Table 1.

Multiplex PCR amplification (50 μl) consisted of 2X KAPA*Taq* Ready Mix DNA polymerase (1.25 U KAPA*Taq* DNA polymerase, 0.4 mM each dNTP, and reaction buffer with $Mg^{2+}$), 20 pmol of each of the 3 pairs of specific primers, 1 μl of DNA template, and sterile $ddH_2O$. PCR thermocycling (C1000™ Thermal Cycler BIO-RAD, Hercules, CA) was performed as follows: 95ºC for 3 minutes; 35 cycles of 95ºC for 30 seconds, 53ºC for 30 seconds and 72ºC for 1 minute; and a final step of 72ºC for 5 minutes. PCR amplicons were analyzed by 2% agarose gel-electrophoresis at 50 volts for 1 hour, visualized with ethidium bromide and recorded using gel documentation system (G:Box HR; Syngene, Cambridge, UK).

**Determination of specificity and sensitivity**

To determine specificity of each primer pair, ~ 2 ng/μl gDNA extracted from adult *A. lumbricoides*, *T. trichiura*, and *N. americanus* singly, and in combinations of two and three, were amplified by multiplex PCR as described above. In addition to STHs, the assay was also performed with other intestinal parasites (see above) for confirming specificity of the primers.

For determining sensitivity of the assay, amplicons of each STH were cloned

Table 1
Oligonucleotide primers used in multiplex PCR for detection of *A. lumbricoides*, *T. trichiura* and *N. americanus*.

| Helminth spp/ DNA target | Accession no. | Primer (5′-3′) | Length (nt) | Product size (bp) |
|---|---|---|---|---|
| *A. lumbricoides* | | | | |
| COI | EU582499 | *Fw*: 5′ GGAGGTTTTTGGGTCTTTGG 3′ | 20 | 192 |
| | | *Rw*: 5′ CCAAACAAGGTAGCCAACCA 3′ | 20 | |
| *T. trichiura* | | | | |
| 18S rDNA | GQ352548 | *Fw*: 5′ CTGCGAGGATTGACAGATCA 3′ | 20 | 498 |
| | | *Rw*: 5′ GTACAAAGGGCAGGGACGTA 3′ | 20 | |
| *N. americanus* | | | | |
| ITS1 | AJ001680 | *Fw*: 5′ ATGCTTGGCAAGAGTCGTTT 3′ | 20 | 330 |
| | | *Rw*: 5′ TGTTGGCGTCCACACATATT 3′ | 20 | |
| hGAPDH | XM005253678 | *Fw*: 5′ GCATCCTGGGCTACACTGAG 3′ | 20 | 150 |
| | | *Rw*: 5′ TGCTGTAGCCAAATTCGTTG 3′ | 20 | |

into pTZ57R/T vector (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions, and DNA inserts were sequencing (AITbiotech, Science Park 1, Singapore). Standards of known copy numbers (1 to $10^6$) of the recombinant plasmids were prepared by 10-fold serial dilution, and subsequently used for determination of sensitivity.

**Data analysis**

Results of multiplex PCR were compared with microscopy examinations to determine specificity and sensitivity of the assay as described by Cornelissen *et al* (2001).

## RESULTS

**Specificity of multiplex PCR**

Multiplex PCR reaction containing specific primers of *A. lumbricoides*, *T. trichiura*, and *N. americanus* was evaluated for specificity with gDNA of single, double and triple STHs. The results of single STH spp showed a single amplicon of 192, 330 and 498 bp for *A. lumbricoides*, *N. americanus*, and *T. trichiura*, respectively, and the presence of the expected amplicons in the mixed samples (Fig 1). The negative control showed no amplification and amplifications of gDNA of other intestinal parasites showed no cross-amplification (data not shown). In addition, control fecal samples (negative for helminth and protozoa) displayed negative results (data not shown).

**Sensitivity of multiplex PCR**

Sequences of PCR fragments of *A. lumbricoides*, *T. trichiura*, and *N. americanus* inserted into pTZ57R/T showed 100% homology with the DNA sequences obtained of their respective STHs using Needle software (EMBOSS program: http://evol.biology.mcmaster.ca/EMBOSS-Pise.html). Standard solutions of recombinant plasmids containing 1-$10^6$ copy numbers were used for examination of sensitivity. Multiplex PCR could detect as little as one copy number of single, double (*A. lumbricoides* and *N. americanus*; *A. lum-*

Table 2
Comparison of microscopic examination and multiplex PCR assay of fecal samples.

| Multiplex PCR | Microscopy | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | | | 120 |
| Single infection | | | |
| *A. lumbricoides* | 38 | 4 | |
| *N. americanus* | 17 | 0 | |
| *T. trichiura* | 9 | 0 | |
| Mixed infection | | | |
| *A. lumbricoides* and *N. americanus* | 4 | 0 | |
| *A. lumbricoides* and *T. trichiura* | 19 | 0 | |
| *N. americanus* and *T. trichiura* | 18 | 0 | |
| All three species | 11 | 0 | |
| Negative | 17 | 19 | 36 |
| Total | 133 | 23 | 156 |

*bricoides* and *T. trichiura*; *N. americanus* and *T. trichiura*), and triple STHs (*A. lumbricoides*, *N. americanus* and *T. trichiura* (data not shown).

**Validation of multiplex PCR for detection of STH infections in fecal samples**

Among the 156 fecal samples examined by Kato-Katz technique, 133 (85%) samples were identified as being positive for STH eggs, with 73 (55%) positive for single infection, 47 (35%) for double infection, 13 (10%) for triple infection and 23 (15%) negative; whereas multiples PCR-based assay of the same 156 fecal samples showed 120 (77%) being positive: 68 (57%) single infection, 41 (34%) double infection, and 11 (9%) triple infection and 36 (23%) negative (Table 2). There were 17 Kato-Katz positive samples that gave negative multiplex PCR results, and 4/23 Kato-Katz negative samples were positive for *A. lumbricoides* with multiplex PCR (Table 2). Using Kato-Katz microscopic technique as the gold standard, the overall sensitivity and specificity of multiplex PCR achieved 87% and 83%, respectively. The sensitivity and specificity in the detection of each STHs was 95% and 93% for *A. lumbricoides*, 77% and 100% for *T. trichiura*, and 89% and 100% for *N. americanus*, respectively. Typical multiplex PCR compared with microscope results of fecal samples are shown in Fig 2.

## DISCUSSION

Until now, microscopic examination to find helminth eggs or larva in fecal samples has been used as the standard method for diagnosing STH infections. Although, this technique is simple and economical but suffers from low sensitivity, especially in cases of light infection. One alternative is PCR assay, which is a highly sensitive technique with high-throughput capacity that can be used for diagnosis of STHs (Verweij *et al*, 2001; Gruijter *et al*, 2005; Leles *et al*, 2009; Areekul *et al*, 2010). More recently, multiplex real-time PCR using fluorogenic probes has been developed for diagnosis of 7 different intestinal

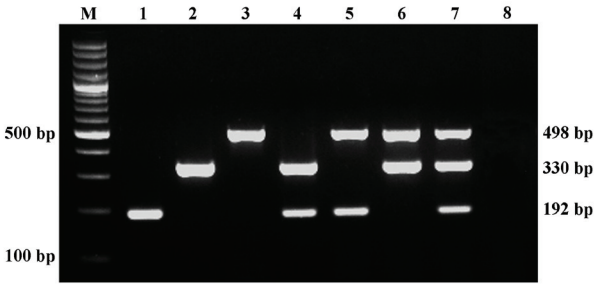


Fig 1– Multiplex PCR detection of STHs. Species-specific amplicons were separated by 2.0% agarose gel-electrophoresis and visualized by ethidium bromide staining. Lane M: GeneRuler™ 100 bp plus DNA ladder; lane 1: *A. lumbricoides*; lane 2: *N. americanus*; lane 3: *T. trichiura*; lane 4: *A. lumbricoides* and *N. americanus*; lane 5: *A. lumbricoides* and *T. trichiura*; lane 6: *N. americanus* and *T. trichiura*; lane 7: *A. lumbricoides*, *N. americanus* and *T. trichiura*; lane 8: negative control.

parasites including STHs, with both high sensitivity and specificity (Taniuchi *et al*, 2011). However, the technique is costly and requires advanced instruments. As a result, in this study we have developed conventional multiplex PCR assay that can simultaneously detect the three major STH infections, *A. lumbricoides*, *T. trichiura*, and *N. americanus*, at a lower cost and requiring only a standard thermal cycler.

The multiplex PCR was capable of detecting as one copy of DNA target of all three STHs, indicating the potential of detecting even a single helminth egg contained in a fecal sample. However, in the real situation, egg numbers of different STHs present in the same sample vary, so that in the STHs presenting a high intensity of eggs will mask the detection of much lower eggs from others by competing PCR amplifying ingredients such as dNTPs, cofactors and *Taq* polymerase.

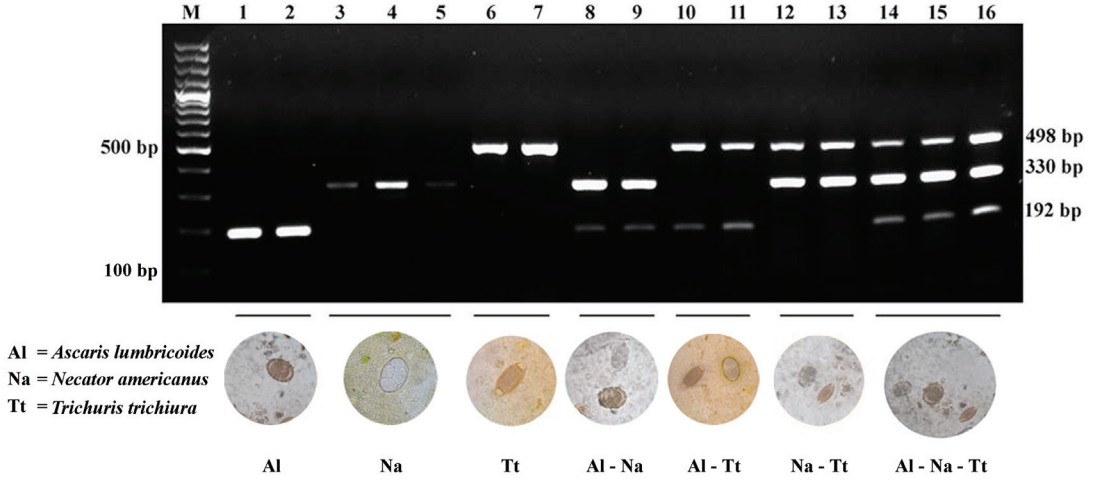


Fig 2–Multiplex PCR detection of STHs from fecal samples. STH DNA was extracted from fecal samples as described in Materials and Methods. Species-specific amplicons were analyzed as described in legend to Fig 1. Lane M: GeneRuler™ 100 bp plus DNA ladder; lanes1-2: *A. lumbricoides*; lanes 3-5: *N. americanus*; lanes 6-7: *T. trichiura*; lanes 8-9: *A. lumbricoides* and *N. americanus*; lanes 10-11: *A. lumbricoides* and *T. trichiura*; lanes 12-13: *N. americanus* and *T. trichiura*; lanes 14-16: *A. lumbricoides*, *N. americanus* and *T. trichiura*.

Users of multiplex PCR should be aware of this intrinsic weakness in the technique particularly when analyzing fecal samples with multiple infections containing eggs predominant of a particular STH because there might fail to detect much other lighter co-infection. Nevertheless, multiplex PCR developed in this study is extremely specific for detecting only the specified target STHs and no other intestinal parasites.

In validation of multiplex PCR with fecal specimens, 13% of microscope-positive samples were negative with multiplex PCR. This might be explained in terms of presence of natural DNA polymerase inhibitors in the fecal samples (Areekul *et al*, 2010), DNA degradation during storage, and inefficient disruption of the helminth eggs. As the multiplex PCR assay using the internal control primers (hGAPDH) was positive (data not shown), degradation of DNA in the store samples is another explanation. However, amplification of DNA samples with hGAPDH primers was positive (data not shown), degradation of sample DNA should not be the cause. Inefficient disruption of the helminth egg is the most likely explanation. Several protocols have been developed, such as ultrasonication, microwaving, boiling, freezing, and their combinations between but have proven unsatisfactory (Iñiguez *et al*, 2002; Traub *et al*, 2004; Harmon *et al*, 2006; Dyachenko *et al*, 2008; Leles *et al*, 2009). Thus, a suitable protocol of egg disruption for DNA preparation needs urgently to be developed.

Surprisingly, 17% of Kato-Katz negative samples were positive for *A. lumbricoides* with multiplex PCR. This implies that the multiplex PCR elicits a very high sensitivity, allowing detection of small numbers of *A. lumbricoides* eggs that are not diagnosed by microscopic examination.

In summary, a multiplex PCR assay for simultaneous detection of three major STHs; *A. lumbricoides*, *T. trichiura*, and *N. americanus*, with high sensitivity and specificity was developed that has the potential to be used in routine diagnosis of fecal samples in the future.

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