OPTIMAL CONDITION FOR MULTIPLEX POLYMERASE CHAIN REACTION (PCR) IN DETECTING *ASCARIS LUMBRICOIDES*, *TRICHURIS TRICHIURA*, AND *NECATOR AMERICANUS* IN PRESERVED STOOL

Received: 12-03-2020 • Accepted: 30-07-2020
<http://dx.doi.org/10.21460/bikdw.v5i1.171>

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ABSTRACT

Background: Multiplex PCR examination is one of the molecular examination methodologies applied to detect soil-transmitted helminth (STH) infection. Optimization of the multiplex PCR method is a complex process, but it is necessary to obtain both a correct detection process and a satisfactory DNA product.

Objective: To determine whether multiplex PCR can be optimized to diagnose STH from Indonesian isolates, and to find the optimal method for detection of *A. lumbricoides*, *T. trichiura*, and *N. americanus* infections in stool that have been stored for 3 years.

Methods: A total of 15 samples were examined, and these samples were previously examined by using a microscopic method, then continued with optimization steps.

Result: The optimal PCR mixture used primers targeting COI gene for *A. lumbricoides*, 18S rDNA for *T. trichiura* and ITS1 for *N. americanus*, 15 µl of Go Taq Green Master Mix, 5 µl of the 3 pairs of primers, 5 µl of DNA template and 4 µl of DdH₂O, and the condition was 30 minutes of 95°C denaturation, 30 second of 53°C annealing and 1 minute of 72°C extension, repeated for 35 cycles.

Conclusion: Multiplex PCR can be optimized for STH detection from Indonesian isolates. The successful detection using the multiplex PCR method was influenced by sample preparation prior to DNA isolation, which includes several steps i.e. homogenization of samples using bead beaters and passing samples on liquid nitrogen rapidly.

Keywords: multiplex polymerase chain reaction, *ascaris lumbricoides*, *trichuris*, *necator americanus*

INTRODUCTION

Soil-transmitted helminth (STH) infection has a worldwide distribution. In 2017, the World Health Organization estimated about 24% of the world population had infected with STH, especially *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, and *Ancylostoma duodenale*.¹ In Asia, the prevalence of STH infection reached 73%, where India and China with the highest proportion.² The prevalence of helminthiasis in Indonesia in 2015 varied by region, ranging from 20 to 86%, with an average of 30%.³

The diagnosis of STH infection is generally conducted by using microscopic and molecular approaches. The later include Polymerase Chain Reaction (PCR), quantitative PCR (qPCR), and multiplex PCR. The PCR method has high sensitivity in detecting parasitic deoxyribonucleic acid (DNA).⁴ In the past decades, multiplex PCR has been used for parasitology evaluation, due to its time efficiency, accuracy and its ability to detect co-infections. These features support an effective diagnosis for daily clinical practice and public health measures.^{5,6} However, some PCR test cycles might

not yield valid results. The multiplex PCR process is influenced by several factors, such as DNA concentration and quality, annealing temperature, MgCl₂ concentration, primer concentration and quality, number of PCR cycles, the concentration of dNTP and other supporting materials.^{7,8}

Previous studies have been conducted to determine the optimal condition to produce reliable results, yet there were only a few studies that exercise this optimisation on Indonesian samples. This research is aimed to determine the optimal condition for the identification of *A. lumbricoides*, *T. trichiura*, and *N. americanus* DNA using multiplex PCR method on preserved stool samples.

METHODS

Material

All stool samples were acquired from the storage facility at the laboratory of parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. The samples were preserved for three years in ethanol 96%, at the temperature -20°C. There are 15 stool samples were obtained from infection cases (of *A. Lumbricoides*, *T. Trichiura*, and hookworm) in Maluku Province, Indonesia, in 2015.

Table 1. Primary nucleotide sequences *A. lumbricoides*, *T. trichiura*, and *N.americanus* used in multiplex PCR.⁹

Helminth spp/DNA target	Forward primer, 5'→3'	Nucleotide length (nt)	Product size (Base pair)
	Reverse primer, 5'→3'		
<i>A. lumbricoides</i> /COI	GGAGGTTTTTGGGTCTTTGG CCAAACAAGGTAGCCAACCA	20	192
<i>T. trichiura</i> /18S rDNA	CTGCGAGGATTGACAGATCA GTACAAAGGGCAGGGACGTA	20	498
<i>N. americanus</i> /ITS1	ATGCTTGGCAAGAGTCGTTT TGTTGGCGTCCACACATATT	20	330

PCR Methods

The diagnostic examination was conducted in several steps. First, the faecal specimens were prepared, either for microscopic stool examination and multiplex PCR testing. For the PCR testing, the faecal deposits were initially emulsified with Lugol solution and further washed with 300 µl H₂O. At the molecular processing stage, the resulted suspensions were centrifuged and further washed two times with the same amount of H₂O. Further PCR steps were performed with methods described by Phuphisut et al.⁹

The second step is DNA isolation from the prepared specimens. All specimen underwent different treatment before DNA isolation procedure, which is (1) direct DNA isolation without pre-treatment, (2) bead-beating homogenisation before DNA isolation (on selected samples), and (3) bead-beating homogenisation and liquid nitrogen treatment before DNA isolation. We used FavorPrep™ Stool DNA Isolation mini kit (Favorgen, Taiwan) for all specimen. The DNA

isolation was performed with PCR components which include 15 µl PCR mixture (GoTaq® Green Master Mix (Promega, USA)). 1µl primers of each parasite (forward and reverse), two concentration (2 µl or 5 µl) of DNA template, and H₂O to adjust the total volume to 30 µl.

On the DNA amplification stage, we initially used a thermocycler with three steps, (1) denaturation at 95°C for 3 minutes; (2) annealing of primers (selected specimen underwent five different temperature, 50°C, 51°C, 52°C, 53°C, 54°C) for 20 seconds; and extension at 72°C extension for 1 minute. We changed the extension time to 5 minutes in the last cycle. Further amplification was conducted with a DNA amplifier machine, using primers targeting *A. lumbricoides*, *T. trichiura* and *N. americanus* DNA (Table 1).

Finally, the amplified specimens further underwent electrophoresis on 2% agarose gel, with 100 V voltage for approximately 30 minutes. The results were visualized through a transilluminator gel documentation machine.

Table 2. The result of a microscopic examination on the preserved stool specimens from Maluku Province, Indonesia (n = 15).

ID	Type of parasite		
	<i>Ascaris lumbricoides</i>	<i>Trichuris trichiura</i>	Hookworm
1	Positive	Positive	Negative
2	Positive	Positive	Negative
3	Positive	Positive	Positive
4	Positive	Positive	Negative
5	Positive	Positive	Positive
6	Positive	Positive	Positive
7	Positive	Positive	Positive
8	Negative	Positive	Negative
9	Positive	Positive	Positive
10	Positive	Positive	Negative
11	Positive	Positive	Negative
12	Positive	Positive	Negative
13	Positive	Positive	Negative
14	Positive	Positive	Positive
15	Negative	Positive	Negative

To determine the optimal DNA concentration yielding the best result, we made several preparations with different DNA concentration from a selected specimen with triple infection, using dilution method (gradual addition of distilled H₂O, with equal volume). This dilution was stopped after the final electrophoresis did not produce positive bands anymore. We further measure the DNA concentration of each specimen with a nanodrop machine.

RESULTS

According to the microscopic examination, among 15 specimens, *A. lumbricoides*, *T. trichiura*, and *N. americanus* were detected in 13 (86.7%), 15 (100%), and 6 (40%) samples subsequently. Mixed infection with *A. lumbricoides*, *T. trichiura* were observed in 13 samples (86.7%), and mixed infection with three parasites were observed in 6 samples (40%). Table 2 describes the microscopic examination of each specimen.

Optimal Annealing Temperature

DNA isolation with five different annealing temperature (50°C, 51°C, 52°C, 53°C, 54°C) was performed on

sample No. 3. Figure 1 shows the electrophoresis result of different annealing temperature, where lane 4 (annealing temperature 53°C) yields the best result for multiplex PCR on a triple infection.

Sample Weight And DNA Template Volume

The sample weight in this study is 5 mg, 30 mg, 50 mg, 100 mg and 200 mg and the templates volume are 2 µl and 5 µl. Initial tests used 5 mg sample and 2 µl DNA template which detected 4 double infections (*A. lumbricoides* and *N. americanus*) and 4 single infections (one *A. lumbricoides*, one *T. trichiura* infection, and two *N. americanus* infections). There are four Necator involvements which did not show on microscopic examination (No. 4, 10, 13, and 15). Further combination of 30 mg samples and 5 µl DNA template could only detect 4 double infections (*A. lumbricoides* and *N. americanus*) and 5 single *A. lumbricoides* infections. The final combination used was 200 mg sample and a 5 µl DNA template. Combined with the pre-treatment, this combination yielded the best detection rate.

Figure 1. Electrophoresis of multiplex PCR products for sample No. 3 (triple infection) in multiplex method with three primers and annealing temperatures at 50°C (lane 1), 51°C (lane 2), 52°C (lane 3), 53°C (lane 4), 54°C (lane 5). M:Marker; N:Negative control; PCR product at 498 Bp showed *Tricuris trichiura*, 330 Bp showed *Necator americanus*, 192 Bp showed *Ascaris lumbricoides*.

Figure 2. Electrophoresis of multiplex PCR product for samples 1-15. The volume sample 5 mg, and 2 μ l DNA template for PCR mixture, with annealing temperature at 53°C. PCR product at 498 Bp showed *Tricuris trichiura*, 330 Bp showed *Necator americanus*, 192 Bp showed *Ascaris lumbricoides*. marker (M), sample 1 (lane 1), sample 2 (lane 2), and sample 3 (lane 3). Sample 4 (lane 4) shows PCR product at 192 Bp and 498 Bp, sample 5 (lane 5) at 330 Bp, sample 6 (lane 6), sample 7 (lane 7) PCR product at 498 Bp, sample 8 (lane 8), sample 9 (lane 9) and sample 10 (lane 10) PCR product at 192 Bp and 498 Bp, sample 11 (lane 11), sample 12 (lane 12) at 192 Bp, sample 13 (lane 13) at 192 Bp and 498 Bp, sample 14 (lane 14), sample 15 (lane 15) PCR product at 498 Bp.

Figure 3. PCR Electrophoresis of PCR product for sample number 5 and 14, with sample volume 50 mg, a template volume 5 μ l for PCR mixture, and annealing temperature at 53°C. PCR product at 330 Bp shows the *Necator americanus*, 192 Bp shows *Ascaris lumbricoides*. markers (M), negative controls (n), sample 5 (lane 1) PCR product at 192 Bp and 330 Bp, samples 14 (lane 2).

Pre-Treatment With Liquid Nitrogen And Bead-Beating Homogenisation

Figure 2 to 5 demonstrates the multiplex PCR results of different pre-treatment at the optimal annealing temperature. Figure 2 shows result from generic DNA isolation without any pre-treatment, where *Trichuris trichiura* positive in 6 samples (No 4, 7, 9, 10, 13 and 15), *Necator americanus* positive in 1 sample (No 5), and *Ascaris lumbricoides* positive in 5 samples (No 4, 9, 10, 12 and 13). Figure 3 shows the result from sample 5 and 14 which underwent pre-treatment with bead-beating homogenisation, where only one sample yielded a positive result for *Necator americanus* and *Ascaris lumbricoides*, although the microscopic examination showed triple infection in both samples. Finally, Figure 4 and 5 show the result of pre-treatment with bead-beating homogenisation and liquid nitrogen for all specimen. The

comparison of positive results between microscopic examination and the optimised multiplex PCR is shown in Table 3 and 4. Using the microscopic examination as the gold standard, multiplex PCR yield 100% sensitivity and 50% specificity for *A. lumbricoides* detection, 46.7% sensitivity for *T. trichiura* detection, and 66.7% sensitivity and 22.2% specificity for *N. americanus* detection (Table 4).

Optimal DNA Concentration

One sample is selected for examining optimal DNA concentration, where initial concentration was 14.7 ng/ μ l. Figure 6 displays the electrophoresis result of each dilution, where the positive results visible on the 4th dilution for all parasites altogether, and further 8th dilution for at least *A. Lumbricoides* and *T. trichiura* detection. Therefore, the minimum DNA concentration of 0.98 ng/ μ L should be fulfilled for optimal detection of three parasites altogether.

Table 3. The result of optimised multiplex PCR on the preserved stool specimens from Maluku Province, Indonesia (n = 15).

ID	Type of parasite		
	<i>Ascaris lumbricoides</i>	<i>Trichuris trichiura</i>	<i>Necator americanus</i>
1	Positive	Negative	Positive
2	Positive	Negative	Positive
3	Positive	Positive	Positive
4	Positive	Negative	Positive
5	Positive	Negative	Negative
6	Positive	Negative	Negative
7	Positive	Positive	Positive
8	Negative	Positive	Positive
9	Positive	Positive	Positive
10	Positive	Positive	Positive
11	Positive	Negative	Positive
12	Positive	Negative	Negative
13	Positive	Positive	Positive
14	Positive	Negative	Positive
15	Positive	Positive	Negative

Figure 4a. Electrophoresis PCR product from samples 1-7, volume for DNA isolation 200 mg, DNA template volume 5 μ l, annealing temperature at 53°C, Homogenization with beat beater and liquid Nitrogen before DNA isolation. PCR product at 498 Bp showed *Tricuris trichiura*, 330 Bp showed *Necator americanus*, 192 Bp showed *Ascaris lumbricoides*. marker(M), negative control (n), positive control (p), sample 1 (lane 1) and sample 2 (lane 2) appears band at 192 Bp and 498 Bp, sample 3 (lane 3) appears band at 192 Bp, 330 Bp and 498 Bp, sample 4 (lane 4) at 192 Bp and 498 Bp, sample 5 (lane 5) and sample 6 (lane 6) band at 192 Bp, sample 7 (lane 7) band at 192 Bp, 330 Bp and 498 Bp.

Figure 4b. Electrophoresis PCR products from sample 8-15 with a volume sample for DNA isolation 200 mg, 5 μ l volume of template DNA, annealing temperature at 53°C and homogenization with beat beater and liquid Nitrogen rapidly before DNA isolation. PCR product at 498 Bp showed *Tricuris trichiura*, at 330 Bp showed *Necator americanus*, at 192 Bp showed *Ascaris lumbricoides*. marker (M), negative control (n), positive control (p), sample 8 (lane 8) shows bands at 330 Bp and 498 Bp, samples 9 and 10 appear (lane 9, lane 10) bands at 192 Bp, 330 Bp and 498 Bp, sample 11 (lane 11) looked band at 192 Bp and 498 Bp, sample 12 (lane 12) looked band at 192 Bp, sample 13 (lane 13) looked band at 192 Bp and 330 Bp, samples 14 (lane 14) appeared band at 192 Bp and 498 Bp, samples 15 (lane 15) looked 192 Bp and 330 Bp.

Table 4. The comparison of microscopic examination and optimised multiplex PCR on the preserved stool specimens from Maluku Province, Indonesia (n = 15).

Type of parasite	Microscopic examination	Multiplex PCR	
		Positive	Negative
<i>Ascaris lumbricoides</i>	Positive	13	0
	Negative	1	1
<i>Trichuris trichiura</i>	Positive	7	8
	Negative	0	0
<i>Necator americanus</i>	Positive	4	2
	Negative	7	2

DISCUSSION

This laboratory study has described the role of optimised multiplex PCR to diagnose STH infections compared to the microscopic examination. Using preserved stool samples might yield a different result with fresh stools, yet it is favourable for PCR examination in Indonesia, where the laboratory facility is limited in certain cities while the infection is spread throughout the country.

Based on our results, there are several findings on the optimal condition to produce an accurate PCR diagnosis, which are annealing temperature, volume of DNA template, the pre-treatment, and minimum DNA concentration. The optimal annealing temperature in this research (53°C) is similar with the previous study with a similar setting in Thailand specimens.⁹ Increasing sample weight and DNA template volume should theoretically improve accuracy. Although there was a decreased detection rate when we add the sample up to 30 mg and the DNA template to 5 µl, we could reach an optimal result with 200 mg sample and 5 µl DNA template. Higher content of the specimen might increase the possibility of finding positive results.

Pre-treatment before DNA isolation procedure with bead-beating homogenisation and liquid nitrogen apparently improved the accuracy. These treatments are supposedly increasing concentration of DNA

extracted, by freezing the cell wall, while cleansing the specimen from any substances which could interfere with the DNA amplification.^{10,11}

Despite the optimal condition for multiplex PCR testing, our result on *T. trichiura* detection was quite low compared to the microscopic examination. Isolation of *T. trichiura* DNA from eggs was difficult due to its sturdy, which might need a longer time to homogenise, heated and centrifugation.¹⁰ Other plausible explanation would be related to the long-term preservation, which conserve the cell wall, except its genetic material. We observed some 'empty' *Trichuris* egg in microscopic examination hence categorised them as positive case. In some cases, the positive microscopic examination might be explained by the possibility of *T. vulpis* infection resembling the *T. trichiura* morphology, but with different genetic material, thus resulted in negative PCR tests.¹²

This study has also determined the minimum DNA concentration of 0.98 ng/µl. Previous research found a more sensitive threshold of 0.001 ng/µl.¹³ However, this sensitivity might be influenced by difference PCR components mixture and facilities.

CONCLUSIONS

Multiplex PCR could be used to diagnose STH infections from the preserved faecal specimen with various detection rate compared to microscopic examination. The optimal settings are (a) annealing

temperature at 53°C, (b) sample weight 200 mg and DNA template volume 5 µl, (c) pre-treatment with bead-beating homogenisation and liquid nitrogen prior to DNA isolation, and (d) minimum DNA concentration of 0.98 ng/µl. Future studies might need to explore other methods to achieve optimal DNA isolation and amplification, especially for *T. trichiura* infection and shorter period of stool specimen preservation. Besides, *T. trichiura* diagnosis with multiplex PCR must be interpreted cautiously with the corresponding microscopic examination to give accurate results.

ACKNOWLEDGEMENTS

The author would like to thank Mr. Martinus Vinsensius Dhona as a previous researcher who permitted to use the samples collected in his research as samples tested in this study.

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