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# IDENTIFICATION OF MICROFILARIAE USING CONVENTIONAL POLYMERASE CHAIN REACTION AND QPCR-HRM

*IDENTIFIKASI MIKROFILARIA MENGGUNAKAN REAKSI POLIMERASE BERANTAI KONVENSIONAL DAN QPCR-HRM*

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## ABSTRACT

**Background:** The presence of filarial worms in the lymph nodes can result in acute symptoms, such as inflammation of the lymph nodes and ducts, particularly in the groin region. As part of the life cycle of filariasis, symptomatic or asymptomatic patients with microfilariae in their blood can transmit the disease via mosquito bites. The inspection of microfilariae that is currently being developed uses Polymerase Chain Reaction (PCR) to carry out a unique DNA search technique. **Purpose:** Identify the type of microfilaria present in filariasis patients using Quantitative PCR High-Resolution Melting (qPCR-HRM) and conventional PCR techniques. **Method:** This study involved the examination of 19 samples using the qPCR-HRM method. Subsequently, the results that were considered positive for microfilaria underwent further testing using conventional PCR. **Result:** The results of the examination using these two methods revealed the presence of Brugia malayi and Wuchereria bancrofti microfilariae with peak melting temperatures ranging from 78.2 – 78.7 °C and 80.8 – 81.2 °C, and fragment sizes of 199 bp and 227 bp, respectively. **Conclusion:** Based on the results of the identification from these two methods, it is evident that microfilariae of Brugia malayi and Wuchereria bancrofti can be detected using both conventional and qPCR-HRM methods.

## ABSTRAK

**Latar belakang:** Adanya cacing filaria pada kelenjar getah bening dapat menimbulkan gejala akut, seperti peradangan pada kelenjar dan saluran getah bening, terutama di daerah pangkal paha. Pada siklus hidupnya, filariasis dapat ditularkan oleh penderita yang didalam darahnya mengandung mikrofilaria baik yang simtomatik ataupun asimtomatik kepada orang lain melalui gigitan nyamuk. Pemeriksaan mikrofilaria yang dikembangkan saat ini yaitu menggunakan pencarian DNA yang spesifik dengan metode reaksi polimerase berantai. **Tujuan:** Mengidentifikasi jenis mikrofilaria yang ditemukan pada pasien filariasis menggunakan metode qPCR-HRM dan PCR konvensional. **Metode:** Penelitian ini menggunakan pemeriksaan 19 sampel dengan metode qPCR-HRM. Selanjutnya, hasil yang dianggap positif mikrofilaria dites kembali menggunakan PCR konvensional. **Hasil:** Hasil pemeriksaan menggunakan kedua metode tersebut menunjukkan adanya mikrofilaria *Brugia malayi* dan *Wuchereria bancrofti* dengan suhu puncak leleh 78,2 – 78,7 °C dan 80,8 – 81,2 °C, serta ukuran fragmen masing-masing sebesar 199 bp dan 227 bp. **Kesimpulan:** Berdasarkan hasil identifikasi kedua metode tersebut, terlihat bahwa mikrofilaria *Brugia malayi* dan *Wuchereria bancrofti* dapat dideteksi menggunakan metode konvensional maupun qPCR-HRM.

## INTRODUCTION

Filariasis is an infectious disease caused by an infection with filarial worms and transmitted by numerous species of mosquitoes. This disease is widespread in both rural and urban areas and can affect people of any age or gender (Santoso and Sitohang, 2017). In Indonesia, filariasis is caused by three types of filarial worms: (1) *Wuchereria bancrofti*, (2) *Brugia malayi*, and (3) *Brugia timori*. *Wuchereria bancrofti* is endemic in Java, Bali, NTB, and Papua, while *Brugia malayi* has the most extensive distribution in

The filarial life cycle can be spread mosquito bites from symptomatic or asymptomatic individuals whose blood contains microfilariae (Global Health, 2015). Regular administration of medication, the establishment of surveillance and laboratory networks, and the advancement of filariasis-related research are all tactics for eliminating filariasis (Ministry of Health, 2015). Health center staff discovered the condition of filariasis patients in Indonesia during the initial screening of finger blood by observing physical symptoms such as swollen feet and nighttime chills; no microfilariae were discovered using microscopic methods. After a microscopic examination, it is necessary to conduct a subsequent test, namely an examination using the *Polymerase Chain Reaction* (PCR) method.

The development of a laboratory diagnosis is one strategy to combat filariasis. This novel technique primarily focuses on detecting and validating microfilariae species. Tracers are used to identify microfilaria using DNA traces and the PCR method. *High-Resolution Melting* (HRM) based on real-time PCR can identify filariasis in blood samples. PCR testing with HRM uses SLX, a specific primer that detects filariasis DNA with high sensitivity (Ihsan *et al*., 2021; Thanchomnang *et al*., 2013). The presence of DNA is then observed using agarose gel at the conclusion of the experiment, utilizing the results of DNA amplification with standard PCR. While HRM based on real-time PCR allows for observations during the reaction, the presence of amplified DNA is visible on the graph that results from the buildup of fluorescence from the probe (marker). Suryaningtyas and Santoso's (2015) study, which identified microfilaria DNA in mosquitoes, similarly utilized conventional PCR. Therefore, it is determined that the PCR approach is sensitive in detecting microfilariae in the mosquito's body (Suryaningtyas and Santoso, 2015). The PCR approach can be used to rapidly detect filariasis in human blood when a mass examination is necessary to identify filariasis (Phuakrod *et al*., 2022). This research should discover one additional test useful for the justification of anti-filarial therapy, with the help of this study, one can monitor the management of filariasis in Purwakarta and provide a foundation for laboratory examinations connected to micro filariasis examination utilizing PCR.

## MATERIAL AND METHOD

The tools used in this study were esco health care PCR tools, a water bath 650, scissors, tweezers, 1.5 mL microtubes, microtube racks, a centrifuge, a vortex, a micropipette, 100 - 1000 μL micro tips (1000 pcs), a timer, BSL level 2 equipment, and marker pens. The materials used included whole blood samples from filariasis-positive and negative patients, cell lysis solution (900 mL), nuclei lysis solution (300 mL), protein precipitation solution (100 mL), isopropanol (300 mL), DNA rehydration solution (100 mL), 2x SensiFAST HRM mix (10 μL), 10 μM forward primer (0.8 μL), 10 μM reverse primer (0.8 μL), and $H_2O$ (16 μL).

### DNA isolation stage

After taking the blood sample, DNA isolation was carried out to obtain genomic DNA using Wizard® Genomic DNA Purification from Promega with the following steps: (1) 300 μL of the sample and 900 μL of cell lysis solution were added into a microcentrifuge tube, stirred and homogenized. The tube was incubated for 10 minutes at room temperature and centrifuged at 15.000 rpm for 3 minutes. The supernatant was removed and the pellet was vortexed. (2) The nuclei lysis solution was added into the tube and mixed. Protein precipitation solution was added and vortexed for 20 seconds, centrifuged at 15.000 rpm for 3 minutes. (3) The supernatant was transferred to a new tube containing 300 μL of isopropanol, mixed, and centrifuged at 15.000 rpm for 1 minute. The supernatant was discarded. (4) 300 μL of 70% ethanol was added into the tube, mixed, and centrifuged at 15.000 rpm for 1 minute. The tube was tilted on a tissue for 15 minutes. (5) 100 μL of DNA rehydration solution was added, followed by incubation in a water bath for 1 hour at 65 °C.

### Real-time *Polymerase Chain Reaction - High Resolution Melting* (PCR-HRM) inspection

After DNA isolation, the screening examination proceeded to the next stage, qPCR-HRM, using a qPCR tool with the SensiFAST HRM Kit reagent. The working steps were as follows: (1) The PCR master mix for HRM was prepared in a tube containing 2x SensiFAST HRM mix (10 μL),10 μM forward primer SLX (0.8 μL),10 μM reverse primer SLX (0.8 μL), $H_2O$ (16 μL), and then 4 μL of sample was added, with a total of 20 μL used. (2) The PCR tube was closed with a lid and placed into the esco health care PCR tool, then the PCR was run. The PCR cycle to obtain the HRM curve must be run under

the following conditions: (1) 1 cycle of 2 minutes pre-incubation at 95 ℃, (2) 40 cycles of 95 ℃ for 5 seconds (denaturation), (3) 60 - 65 ℃ for 10 seconds (annealing), and (4) 72 ℃ for 5 - 20 seconds (extension). After the reaction was complete, a melting curve analysis was carried out (Thanchomnang *et al.*, 2013). Finally, the examination was proceeded with the conventional PCR method, specifically the DNA amplification stage.

### Conventional *Polymerase Chain Reaction* (PCR) examination

Target DNA amplification is aimed to increase the amount of target DNA present so that it could be detected by electrophoresis. DNA amplification was carried out with the help of a thermocycler, better known as a PCR tool. The amplification process of conventional methods always includes positive samples known to contain microfilariae and negative controls. The reaction mixture was prepared with the composition for one reaction, which consisted of 6.25 µL Promega Go Taq® Green Master Mix, 0.5 µL WSSV270 primer, 0.5 µL WSSV345 primer, and 4.25 µL NFW. The reaction mixture was homogenized by centrifugation. Then, 11.5 µL of the reaction mixture was distributed to 0.2 mL microtubes for samples, positive control and negative control.  1 µL of NFW was added to the microtube containing the negative sample.

Whereas, 1 µL of sample DNA template was added to sample microtubes according to each code and 1 µL of positive microfilariae DNA was added to the positive control microtube. All tubes were vortexed and homogenized by centrifugation, then put into the PCR machine, and the machine was run with the following temperature settings, namely pre-amplification, denaturation at 94 ℃ (4 minutes), annealing at 55 ℃ (1 minute), and extension at 72 ℃ (2 minutes) for 1 cycle. Subsequently, denaturation at 94 ℃ (1 minute), annealing at 55 ℃ (1 minute), and extension at 72 ℃ (2 minutes) were repeated for 30 cycles. Then, the final extension was at 72 ℃ (5 minutes) for 1 cycle, followed by an end hold at 4 ℃. The band sizes obtained were 199 bp for *Brugia malayi* and 227 bp for *Wuchereria bancrofti*.

### RESULT

### Identification using real-time *Polymerase Chain Reaction* (PCR)

The analysis of 19 blood samples using real-time PCR revealed the existence of four samples testing positive for microfilaria. The *Temperature Melting* (TM) results obtained from the sample examination are illustrated in Figure 1. The TM value for sample number 9 (Fig. 2c) was recorded at 78.8 ℃, as demonstrated by the melting curve image. Sample 9 included *B. malayi*

microfilariae DNA (Fig. 2a), as evidenced by the identical result to the positive control for *B. malayi* microfilariae. Then, sample number 13 (Fig. 2d) exhibited a TM value of 80.5 ℃, matching the positive control for *W. bancrofti* (Fig. 2b). It was assumed that this sample contained microfilariae DNA from *W. bancrofti*.

Moreover, sample number 18 (Fig. 2e) yielded a TM result of 80.3 ℃, indicating the presence of *W. bancrofti* microfilariae DNA. Meanwhile, sample number 19 (Fig. 2f), with a TM result of 78.2 ℃, contained *B. malayi* microfilariae DNA. In addition to the melting curve results, there were also  amplification results for the four positive samples, as illustrated in Figure 2. The following are the results of the amplification of samples containing microfilariae DNA.

Based on the amplification results of the four samples suspected containing microfilariae DNA, there were amplification with a *Cycle Threshold* (CT) of 18 for samples (Fig. 3a), (Fig. 3b), and (Fig. 3c), and a CT of 27 for sample (Fig. 3f). These amplification results indicated that the four obtained samples contained microfilariae DNA.

### Identification with conventional *Polymerase Chain Reaction* (PCR)

In order to avoid false positives with real-time PCR, more tests were conducted using conventional PCR. The results of the conventional PCR are shown in Figure 1.
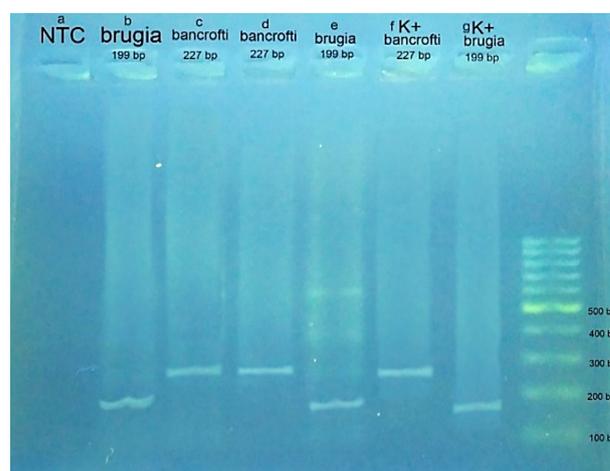


**Figure 1.** Electrophoresis *Polymerase Chain Reaction* (PCR) results: (a) *No Template Control* (NTC), (b) Sample result number 9, (c) Sample result number 13, (d) Sample result number 18, (e) Sample result number 19, (f) Positive control of *W. bancrofti*, and (g) Positive control of *B. malayi*

Based on the preceding results, it can be concluded that the electrophoresis PCR results were identical to the results of the qPCR, with samples number 9 and 19 indicating the presence of *B. malayi* microfilariae DNA, and samples number 13 and 18 showing the presence of *W. bancrofti* DNA. Based on the initial confirmation results, it can be stated that qPCR did not provide any false-positive results.
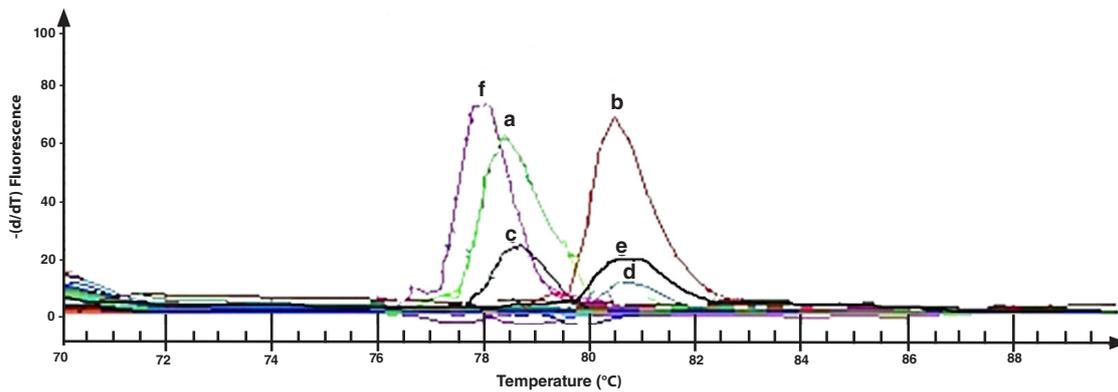
**Figure 2.** Melting peak results from sample examination: (a) *Brugia malayi* positive control result (TM 78.5 °C), (b) *W. bancrofti* positive control result (TM 80.6 °C), (c) Result of sample number 9 (TM 78.8 °C), (d) Result of sample number 13 (TM 80.5 °C), (e) Result of sample number 18 (TM 80.3 °C), and (f) Result of sample number 19 (TM 78.2 °C)
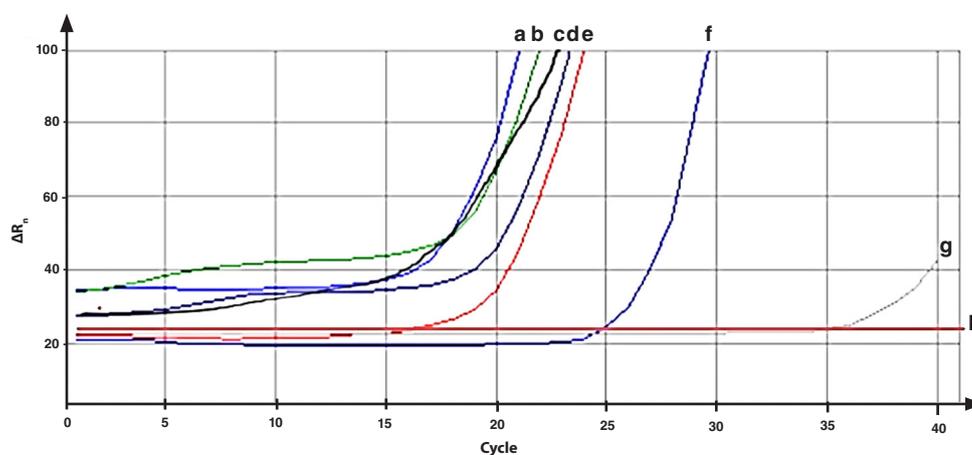


**Figure 3.** Amplification results: (a) Sample number 9, (b) Sample number 13, (c) Sample number 18, (d) Positive control of *Brugia malayi,* (e) Positive control of *W. bancrofti,* (f) Sample number 19, (g) The result of the sample amplification is negative. (h) Amplification results for *No Template Control* (NTC)

## DISCUSSION

The real-time *Polymerase Chain Reaction* (PCR) results of this study are consistent with those of Thanchomnan's (2013) study, which suggested that qPCR-HRM data could be utilized to identify the type of DNA filariasis. It also reveals comparable melting temperature findings (Rojas *et al*., 2015; Thanchomnang *et al*., 2013). Similar to an earlier study, the results of TM *Brugia malayi* are in the lower range compared to *W. bancrofti* and are not particularly significant (Scavo *et al*., 2022; Wongkamchai *et al*., 2014).

Based on the diagnostic state of the sample, the CT value is used to determine whether the amplification results of this investigation are positive or negative. The amount of template present at the beginning of the amplification process mostly determines the CT value. CT is primarily influenced by the number of molds present at the start of the amplification process (Alhassan *et al*., 2016). CT values for all samples range from 18.1 to 27.1. *W. bancrofti* and *Brugia malayi* were amplified based on the range of CT values. In this study's

sample, a CT value greater than 30 indicates that the sample is negative for *W. bancrofti* and *Brugia malayi* infections. Assuming that a large number of molds are available at the start of the reaction, relatively few cycles of amplification are required for the product to exhibit fluorescence, resulting in a low CT value for this reaction. In addition to measuring the amplification, we must also measure the primer so that it can be read by the PCR instrument (Lau *et al*., 2021).

Alternatively, if few resources are available, the CT value for this reaction will be high. According to a previous study, the larger the number of microfilariae in the extracted DNA sample, the lower the CT value. In contrast, the CT value rises as the microfilariae density in the extracted DNA samples diminishes. The greater the amount of *B. malayi* and *W. bancrofti* microfilariae in a sample, the greater the amount of DNA from these two worms. This allows for faster amplification, resulting in a lower CT value (Ta-Tang *et al*., 2022). This was also the case in previous studies, where smaller CT values indicated high sensitivity for real-time PCR results (Ramakrishna U Rao *et al*., 2006).

Using real-time PCR, filariasis DNA was found in both chronic and previously treated individuals. This aligns with the findings of a 2006 study from Ramakrishna University, which concluded that real-time PCR could detect persistent infection or parasitic remains that are undetectable by conventional techniques, such as microscopic methods or dissection (mosquito), in patients who have been treated up to ten times (Loymek *et al.*, 2021; Ramakrishna U. Rao *et al.*, 2006). The results of this real-time PCR were also supported by the research of Ferlianti (2012), which concluded that the qPCR examination technique was especially suited for identifying filariasis patients following treatment (Ferlianti, 2012). qPCR has the ability to discriminate filariasis kinds, which is useful for prevention and control surveys. Additionally, this technology is simpler to employ than the majority of other DNA-based methods (Nonsaithong *et al.*, 2018; Wongkamchai *et al.*, 2014).

Based on the results of PCR electrophoresis, it can be inferred that qPCR does not produce any false-positive results. Sirichit Wongkamchai corroborated the results of real-time PCR with PCR electrophoresis, obtaining microfilaria with both methods (Wongkamchai *et al.*, 2014). After receiving negative results from a microscopic examination, confirmation investigations were performed utilizing various microfilariae examinations (Ramakrishna U. Rao *et al.*, 2006). Prior research has demonstrated that the sensitivity of real-time PCR and electrophoresis PCR may detect microfilaria DNA in the bodies of filariasis patients. Previous research reported that qPCR or PCR electrophoresis has a sensitivity that can read microfilariae DNA found in filariasis patients. Later, PCR electrophoresis and qPCR were developed to identify many filarial species, and PCR-based assays directly indicate active infection (Nirwan *et al.*, 2022; Rahmah *et al.*, 2010). This method is suited for filariasis surveillance because PCR-based research has led to numerous scientific advancements in Medical Laboratory Technology/Biotechnology. Using this technique, the variables that impede filariasis testing can be circumvented (Majawati, 2014; Suarez *et al*, 2022; Zhong *et al.*, 2017). In the long term, continuous use of the PCR method can potentially help surveillance strategy for filariasis elimination programs in Indonesia by using research samples, namely mosquitoes and flies (Dieki *et al.*, 2022; McPherson *et al.*, 2022).

## CONCLUSION

Examination using the *Polymerase Chain Reaction* (PCR) method revealed microfilariae DNA in blood samples and identified two types of microfilariae, *B. malayi* and *W. bancrofti*, which had not yet been identified using the microscopic methods. The PCR technique can also detect filaria DNA in post-treatment patients, making it more suitable for determining the efficacy of lymphatic filariasis elimination.

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