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Comparison of Microscopic to PCR for Detecting Microfilaria in 21 Lymphatic Filariasis Patients Treated with Diethylcarbamazine

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Background: Lymphatic filariasis (LF) marked by the World Health Organization (WHO) as one of seventeen Neglected Tropical Disease (NTD) which can be eliminated. Giemsa-stained blood smear remained as the standard method to detect microfilariae, albeit this method has many weaknesses. The PCR method has long been proposed to replace it, but PCR is seldom used in a routine examination. **Objective:** This study aimed to examine the sensitivity and specificity of standard examination compared to PCR in post-therapy subjects. **Methods:** As many as 21 subjects, who had received Diethylcarbamazine (DEC) for 10 days, were enrolled in the study. The capillary blood sample was taken 6 months after the therapy. Half of the blood samples was examined using Giemsa –stained blood smear, and the other half using the PCR. **Results:** From 6 positive samples, the PCR only confirmed 4 of them. The sensitivity of the blood smear was 100% and the specificity was 88%. **Conclusion:** The Giemsa-stained capillary blood smear has a better sensitivity and specificity compared to the PCR. Thus, it remains the gold standard to check microfilaria in routine field examination. A PCR can be used as an alternative.

Keywords: lymphatic filariasis, microfilaria, blood smear, microscopy, PCR

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INTRODUCTION

Filariasis or elephantiasis is a chronic infectious disease caused by the filarial worm which attacks lymph nodes. The microfilariae may damage the lymphatic system, causing a swelling of the hands, feet, mammary gland, and scrotum. Therefore, causing a lifelong disability. Moreover, a social stigma may be attached to the patient and the family.[1]It is a disease transmitted by mosquitoes which contributes to a decrease in human productivity, thus causing economic losses for the country. More than 130 million people in 83 countries had been infected with endemic microfilaria. Filariasis was recorded in 43 countries out of 50 countries with a slow economic growth.[2] Several studies in India, Brazil, Sri Lanka, and the Philippines concluded that the money spent for the filariasis acute stage treatment reached 60 to 85 million US Dollar.[3]A 1998 study conducted by the Ministry of Health in collaboration with University Indonesia School of Public Health showed the cost of filariasis patient care per year was about 17.8% of the total family expenditure or as much as 32.3% of the cost of a family meal.

Filariasis is endemic in almost all of Indonesia regions. Filariasis is a serious community health problem with a high morbidity, especially in the lower middle-class people. Filariasis endemic areas are generally low-lying, mainly rural, coastal, inland, rice fields, marshes, and forests. Bancroft'sfilariasis exists in Sumatra, Java, Kalimantan, Sulawesi, Nusa Tenggara, Maluku and Papua. *Wuchereria bancrofti* endemic areas are divided into rural and urban types based on the transmitting vectors. Wuchereria of the rural type is found mainly in Papua and Nusa Tenggara with Anopheles, Culex, and Aedes as the vectors. Meanwhile, the urban type can be found in Jakarta, Bekasi, Tangerang, Semarang, Pekalongan and Lebak in a seedy and densely populated areas with many puddles of dirty water, with *Culex quinquefasciatus* as the vector. *Brugia malayi* is scattered in Sumatra, Kalimantan, Sulawesi and on several islands of Maluku, while *Brugia timori* is scattered on the islands of Flores, Alor, Rote, Timor, and Sumba. Filariasis endemic areas are areas with microfilaria rate more or equal to 1%. The 2009 data from the Indonesian Ministry of Health

proposed that there were as many as 11,914 people with elephantiasis and there were 337 endemic districts.[4-6]

In 1997, the World Health Organization (WHO) called for an eradication of the lymphatic filariasis (LF). In 2000, the WHO launched a global program for elimination of LF (GPELF) project, aiming to eradicate filariasis in 2020.[5] Diethylcarbamazine (DEC) was first introduced in 1947. However, the filariasis prevalence in Asia, including Indonesia, were still high (49%) when compared to other regions worldwide.[7]

The filariasis eradication covers two aspects: the control of the transmission and the treatment of patients disability. If the transmission can be controlled, a new case will cease to emerge. The transmission control can be done by treating the filariasis patients, individually or in mass, as well as doing a vector control.[8]

A Giemsa-stained capillary blood smear is a standard method for detecting microfilaria. The smear can be a thick or a thin smear. The microscopic method has a high sensitivity and a low specificity because it depends on the skill of the laboratory examiner, the amount of blood available, the time of blood sampling, and the blood retrieval technique.[9] Microfilariae may not be detected in the early and in the late phase of infection because microfilaria disappeared from the bloodstream. In addition, the microscopic technique is not useful in a filariasis infection without microfilaria and when the microfilaria density is low after DEC administration.[10] Unfortunately, a serological examination has a low sensitivity and specificity, as this technique cannot distinguish an acute infection to a cured one.[11]
A molecular examination, such as a conventional PCR and a real-time q-PCR, has been developed to replace the existing examination technique. But, PCR is still rarely used for a routine examination. Our study aimed to compare the sensitivity and specificity of microscopic techniques to PCR in detecting microfilaria in filariasis patients treated with DEC.

According to the WHO, DEC and Albendazole are the standard regiments on areas with tissue nematodes. They kill the adult and the young worms. After 10 days of DEC, the density of the microfilariae is usually low (less than the microscopic threshold), so that the microscopic examination will give a false negative. A PCR is performed to detect the false negative in order to monitor the therapeutic efficacy.

MATERIALS AND METHOD

We recruited the sample from the lymphatic filariasis March 2014 report of the Balangan District Department of Health, South Kalimantan. The district was endemic for microfilaria. The lymphatic filariasis patient was diagnosed if the Giemsa-stained blood smear examination positive for microfilaria through microscopic examination. The patients received 10 days DEC and albendazole therapy, per oral. We contacted the patients in one of the villages in the district, Gullinggang village. The patients were included in our study if they agreed to participate and signed an informed consent form.

The 1 ml capillary blood sampling was carried out 6 months after DEC administration. The blood sample was stored in vacutainer tubes containing EDTA. The capillary blood sample was taken with a 60 µlmicropipette to make a blood smear preparation by making 3 lines, each line contains 20 µl. Next, it was stained using Giemsa staining. A DNA isolation from 200 µl of blood was done using QIAamp® DNA Mini Kit (Qiagen, Germany) according to Qiagen manual. The end result is a 200 µl DNA. The forward and reverse primer sequences for *Brugia malayi* is Hha1 F 5'-GCG AAA CAT TTC AGC ATC-3 ', 5'-GCG Hha1 R TTA CAA AAC ACA ATT AAA GC-3' (AIT biotech), commercially purchased.[12]The total volume of blood for the PCR was 50 µl (KAPA Taq Extra Hotstart of KAPA BIOSYSTEMS). It was conducted in this sequence: initial denaturation 95°C for 3 minutes, denaturation 95°C for 30 seconds, annealing 57°C for 30 seconds, extension 72°C for 1 minute, final extension 72°C for 1 minute, and the last was a hold for 4°C. The DNA product was visualized using 2% agarose gel.

RESULT

The microfilariae density was checked and recorded. The goal of the PCR was to perform gene amplification in HhaI (**Table 1**). In **Figure 1**, column G, J and P (sample no. 6, 9 and 15) gave a positive result for DNA *Brugia malayi* at 322 bp. In **Figure 2**, column D also gave positive results in 322 bp. The microscopic examination sensitivity was 100% and specificity reached 88%.

DISCUSSION

Wuchereria bancrofti is the most prevalent cause of lymphatic filariasis cases in the world. But,around 70% of lymphatic filariasis cases in Indonesia were caused by Brugia malayi. The worm can be detected using 60µl of capillary blood by staining it with Giemsa to microscopically calculate the density of microfilariae (the number of the young worm). However, sometimes the microscopic examination gives a negative result on microfilariae, especially when the presence were only in a small amount.[12]A polymerase chain

reaction (PCR) can overcome this weakness. A PCR examination using the parasites DNA have been reproduced into thousands of copies and gives a positive outcome when the microscope examination cannot detect the small amount of worm.

Table 1. Microscopic and PCR Examination Results

No.	Sample Code	Microfilariae Density per 60 mm^3	PCR
1	PT_ 1_GG_01_A	0	-
2	PT_ 1_GG_02_A	0	-
3	PT_ 1_GG_03_A	0	-
4	PT_ 1_GG_04_A	0	-
5	PT_ 1_GG_05_A	0	-
6	PT_ 1_GG_06_A	13	+
7	PT_ 1_GG_07_A	0	-
8	PT_ 1_GG_08_A	0	-
9	PT_ 1_GG_09_A	100	+
10	PT_ 1_GG_10_A	0	-
11	PT_ 1_GG_11_A	0	-
12	PT_ 1_GG_12_A	0	-
13	PT_ 1_GG_04_B	0	-
14	PT_ 1_GG_05_B	0	-
15	PT_ 1_GG_06_B	18	+
16	PT_ 1_GG_07_B	0	-
17	PT_ 1_GG_08_B	1	-
18	PT_ 1_GG_09_B	363	+
19	PT_ 1_GG_10_B	0	-
20	PT_ 1_GG_11_B	2	-
21	PT_ 1_GG_11_B	0	-

Table 2. Microscopic Sensitivity and Specificity

		PCR		Total
		+	-	
Microscopic	+	4	2	6
	-	0	15	15
Total		4	17	21

Sensitivity = 4/4 = 100%
Specificity = 15/17 = 88%

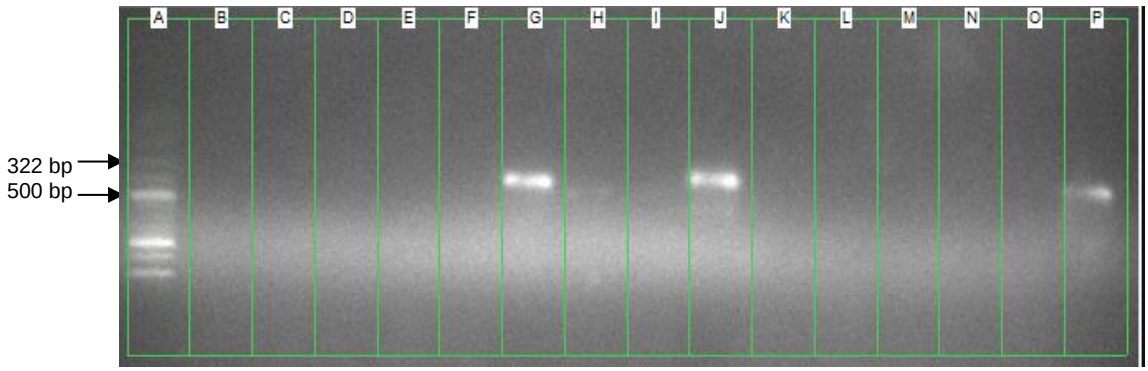

Figure 1.
PCR result of the sample 1 to 15
Column A showed DNA marker ladder 100 bp, column B to P showed the sample 1 to 15

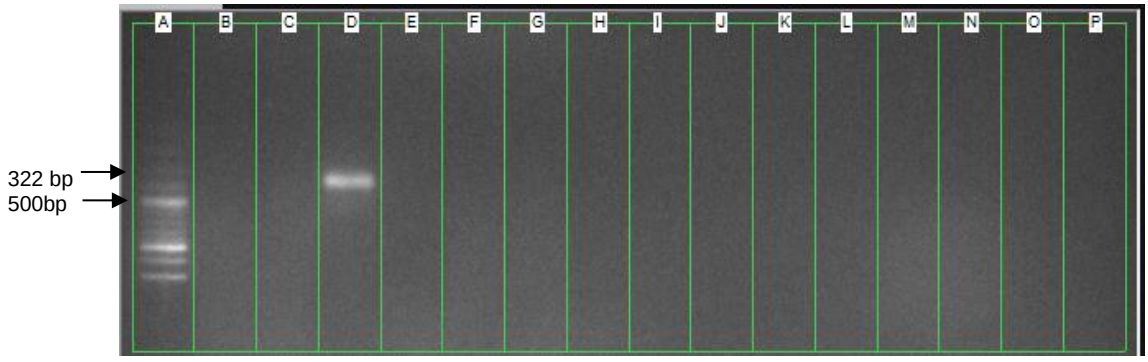

Figure 2.
PCR result of the sample 16 to 21
Column A showed DNA marker ladder 100 bp, column B to G showed the sample 1 to 15, column H to P were blank (did not contain a sample)

In the PCR examination, the HhaI sequence at 322 bp for *Brugia malayi* is positive in specimens with microfilariae. But, the HhaI repeat is arranged in tandem and have more than 30,000 copies per genome haploid and have a recognition site of Alu I and Rsa I, so that a 644 bp dimer and a trimer at 966 bp are usually formed.[13-16]

The sample 17 and 20 were positive microscopically, but negative on the PCR tests. Although it is theoretically possible that PCR could detect microfilariae in small amounts, a PCR also has its weaknesses which cause it to run sub-optimally. The repeated cycle of thawing-freezing may result in a decrease in the amount of DNA. Moreover, the DNA isolation storage technique may be faulty. Some of these obstacles can be overcome by dividing the sample into aliquots (dividing the sample into several small tubes). Therefore, for a test, only one small tube is needed. Thus, it will not compromise the DNA overall results.

We found that the microscopic examination sensitivity was 100%. The specificity was 88%.

CONCLUSION

Microscopic examination is the gold standard to detect *Brugia malayi*. But, PCR is useful as an alternative examination to confirm the negative test results on when the microscopic examination is negative.

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