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Aedes aegypti as potential vector of filariasis in Pekalongan, Central Java Province, Indonesia

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ABSTRACT

Background: The filariasis elimination program in Indonesia has been conducted, but new cases and some chronic cases are still often found.
Objective: This study aims to determine levels of endemicity and to identify filarial worm species in filariasis cases and s and their surrounding communities by using microscopic examination, polymerase chain reaction (PCR), and to examine levels of infection in vectors mosquito by surgery and PCR. Also to to determine that *Ae. aegypti* can act as vector of filariasis.
Methods: This study was conducted at 10 locations in Pekalongan Regency, Central Java Province, with a cross sectional design. Intravenous blood sampling was conducted on 102 respondents consisting of 10 elephantiasis patients and 92 non-elephantiasis patients at night, starting at 8 pm, then examined microscopically and PCR. Mosquitoes in this study were collected by using a human landing collection method for 12 hours from 6 pm to 6 am by volunteers. Artificial infection of microfilaria *W. bancrofti* was held against *Cx. quinquefasciatus* and *Ae.aegypti* from laboratory collection.
Results: Results of this study found that there were 5.729 of mosquitos, consisting of 8 species, namely *Culex quinquefasciatus*, *Culex vishnui*, *Culex tritaeniorhynchus*, *Aedes aegypti*, *Aedes albopictus*, *Anopheles subpictus*, *Anopheles vagus*, and *Armigeres kesseli*. Microfilarial (mf) rate was 0.89%, and and the blood PCR showed infection rate of 3.92% and the blood PCR showed infection rate of 3.92%. No larva was found in female mosquito dissection. The PCR results showed that the infection rate was 9.10% in *Ae. aegypty* pool respectively. Artificial infection results was negative both dissecting microscopis and PCR.
Conclusion: This study revealed that the locations were low of filariasis endemicity. The mf rate was less than 1%, and there was a moderate density to high density of microfilaria in the patients. The low level of infection rates in mosquito is suggested as an alert to its potential transmission.

Latar Belakang: *Program eliminasi filariasis di Indonesia telah dilakukan, namun masih ditemukan kasus baru, dan menujukkan peningkatan kasus kronis.*
Tujuan: *Penelitian bertujuan untuk menjelaskan tingkat endemisitas dan mengidentifikasi spesies cacing filaria pada penderita filariasis kronis dan penduduk di sekitarnya dengan pemeriksaan mikroskopis dan*

PCR, serta mengetahui tingkat infeksi nyamuk vector dengan pemeriksaan bedah dan PCR.

Metode: *Penelitian dilakukan di Kabupaten Pekalongan Provinsi Jawa Tengah di 10 lokasi dengan desain cross sectional. Pengambilan darah intravena dilakukan pada malam hari mulai pukul 20.00 WIB terhadap 102 responden, terdiri atas 10 penderita elefantiasis dan 92 non elefantiasis dan diperiksa secara mikroskopis dan PCR. Nyamuk ditangkap dengan metode human landing collection selama 12 jam mulai pukul 18.00 sampai 06.00 oleh relawan. Infeksi buatan mikrofilaria W. bancrofti dilakukan terhadap Cx. quinquefasciatus dan Ae.aegypti dari koleksi laboratorium.*

Hasil: *Hasil tangkapan nyamuk adalah 5729 ekor dengan 8 spesies yaitu Culex quinquefasciatus, Culex vishnui, Culex tritaeniorhynchus, Aedes aegypti, Aedes albopictus, Anopheles subpictus, Anopheles vagus, dan Armigeres kesseli. Ditemukan microfilaria rate 0.89% dengan kepadatan mikrofilaria 416.67 mf/mL. Hasil PCR darah adalah infection rate 3.92%. Hasil pembedahan pada nyamuk betina adalah negatif. Hasil PCR adalah pool nyamuk Cx. quinquefasciatus dengan infection rate 0.89% dan pool Ae. aegypti dengan infection rate 9.10%.*

Kesimpulan: *Hasil penelitian menunjukkan endemisitas di lokasi penelitian rendah dengan mf rate <1%, dan kepadatan mikrofilaria sedang sampai tinggi. Tingkat infeksi pada nyamuk yang rendah tetap mengharapkan kewaspadaan terhadap potensi penularannya.*

INTRODUCTION

Indonesia is located in a tropical area which has a lot of natural resources, including rich varieties of flora and fauna. The climate and tropical environment in Indonesia are suitable for breeding of mosquitoes which can act as vectors for various diseases.[1] 1One of the diseases transmitted by mosquitoes is lymphatic filariasis. Filariasis is caused by blood and tissue nematode worms. These worms live in the lymphatic system for many years and cause a pathology in a form of elephantiasis.[2] One of lymphatic filariasis is caused by *Wuchereria bancrofti* (*W. bancrofti*), and is transmitted by the *Culex quinquefasciatus* (*Cx. quinquefasciatus*) mosquito.[3] One of them happened in Pekalongan, and its periodicity is nocturnal.[4]

Filariasis cases in Indonesia are still high. In Indonesia, in 2019 there were 10.758 cases of filariasis spread across 34 provinces; this report is higher than the previous year.[5] Data of the Central Java Province of Indonesia in 2018 reported that there were 397 chronic filariasis cases spread across 34 districts, including 9 districts as filariasis endemic areas.[6] The filariasis elimination program in Indonesia has been conducted based on the 2000 global agreement, namely "The Global Goal of Elimination of Lymphatic Filariasis as a Public Health Problem the year 2020" which is realization of the WHO resolution in 1997. The elimination program is conducted through two pillars of activities, namely providing filariasis mass prevention drugs to all residents in filariasis endemic districts, called PPOM (*Pemberian Obat Pencegahan Massa*), and managing filariasis clinical cases to prevent and reduce disability.[7]

Chronic sufferers are a source of transmission of filariasis if their blood contains microfilariae which can be detected by microscopic examination of the smear. Microscopic blood examination often shows false negative results in the prepatent condition. Therefore, it is necessary to conduct molecular examinations by PCR. The transmission can be also confirmed by adiscovery of a mosquito containing stage 3 larvae in its body. Examination of mosquitoes can performed surgery by using a microscope and also a PCR examination.[8]

Previous studies have obtained information that microfilariae are still found in the peripheral blood circulation at 6 am 4. On the other hand, at the same hour, the *Aedes aegypti* (*Ae. aegypti*) mosquito starts biting humans.[9] These two phenomenon raise the question of whether there is an interaction between the two, so that the *Ae. Aegypti* mosquito can act as a vector for filariasis.

This study aims to determine levels of endemicity and identify species of filarial worms in filariasis cases and its surroundings by microscopic examination and PCR. to determine levels of mosquito vector infection with microscopic surgery and PCR, and to determine that *Ae. aegypti* can act as vector of filariasis.

METHODS

Study design, time and place of research

This study was conducted in Pekalongan Regency on March 2019 in 10 locations according to case data for chronic filariasis/ elephantiasis. This study was a cross sectional design. Subjects of this study were chronic elephantiasis patients and non-elephantiasis people around them.[10] This study was approved by the Medical and Health Research Ethics Committee, with No. KE/FK/1113/EC/2018.

Night Intravenous Blood sampling, microscopic and PCR examination

Blood sampling were conducted intravenously, at night starting at 8 pm.[4,7,10] The Blood was collected at night starting at 8pm in one elephantiasis patient and 9 non-elephantiasis people in the vicinity.[10] The Blood was collected for 10 nights at a different location. The blood was then made as a blood slide, and some of others were put into an EDTA tube for PCR examination. During transportation , blood was stored in a cold holding bag for less than 12 hours.

Microscopic examination was conducted in a laboratory. The dried blood slide was then haemolyzed with distilled water, fixed with absolute methanol, and stained with Giemsa 1:9. The dried slide was examined by a light microscope with weak to medium magnification.[10]

The mf rate (%) was calculated by formula:

$$\frac{number\ of\ positive\ samples}{number\ of\ all\ samples} x100$$

while the microfilaria density (permL) was calculated by formula:

$$\frac{number\ of\ microfilaria\ found\ in\ slide}{number\ of\ slide} X16.67$$

PCR assay was performed on both blood and mosquitoes by the same procedure. Isolation of DNA used a kit and was based on the Geneaid for tissue procedure. The obtained genomic DNA (gDNA) was stored in a refrigerator at 4°C before running the PCR. The PCR examination was with Wuchereria bancrofti primers (Ssp I F 5'-CGT GAT GGC ATC AAA GTA GCG-3 ', and Ssp I R 5'-CCC TCA CTT ACC ATA AGA CAAC-3').[11] The PCR assay was performed by setting as follow:

Pre denaturation	94°C 5 minutes	
Denaturing	94°C 1 minute	40 cycles
Annealing	56°C 1 minute	
Extension	72°C 1 minute	
Final extension	72°C 10 minutes	

The PCR results were then performed as electrophoresis and viewed under UV light to determine which bands were formed. *Wuchereria bancrofti* was considered to be positive if the band was formed at 188bp.[11] Data is presented in descriptive form.

Mosquitos collection, filarial worms and PCR examination

The mosquitoes were captured by using a human landing collection method for 12 hours from 6pm to 6am by volunteers. Next, the captured mosquitoes were identified based a guidance of the Rattanirithikul identification key book.[12]

All female mosquitoes were dissected by using a pool technique. 5 to 10 mosquitoes of same species from one location were placed on a glass object, and their wings and legs were removed. Surgery was performed in a phosphate buffered saline (PBS) solution by using a dissecting microscope. Moving objects such as sausages or long objects such as worms were counted and recorded.

The larval density (permL) was calculated by formula: $\frac{number\ of\ larva\ found\ in\ pool}{number\ of\ pool} X16.67$

PCR assay was performed similarly as PCR assay for blood procedure.

Artificial infection

Artificial infection was carried out on 4 groups of mosquitoes, using the waterbath at 37°C. Three groups were *Cx quinquefasciatus* and 1 group was *Ae. aegypti*. Dissecting and PCR examination on 4 groups of test mosquitoes held on day 8th and 14th of infection.

RESULTS

Blood was collected from 102 respondents consisting of 10 elephantiasis patients and 92

non-elephantiasis volunteers around them. Microscopically, 1 positive slide of *Wuchereria bancrofti* was found from the non-elephantiasis with mf rate of 0.89% and with a mf density of 416.67 mf/mL. PCR assay was performed on all blood samples, both positive and negative microfilariae. The results of the PCR assay showed that 4 were positive of 102 samples (infection rate of 1.96%) as can be seen in table 1.

Table 1. Microfilaria rate and PCR infection rate of elephantiasis and non-elephantiasis blood samples in 10 locations in Pekalongan Regency, Central Java Province, Indonesia

No	Location Code	Number of sample		Microscopic (+)		PCR (+)	
		Elephantiasis	Non elephantiasis	Elephantiasis	Non elephantiasis	Elephantiasis	Non elephantiasis
1	A	1	10	0	1	0	1
2	B	1	9	0	0	0	0
3	C	1	9	0	0	0	0
4	D	1	9	0	0	0	0
5	E	1	10	0	0	0	0
6	F	1	9	0	0	0	0
7	G	1	9	0	0	0	1
8	H	1	9	0	0	0	0
9	I	1	9	0	0	0	0
10	J	1	9	0	0	0	0
Σ		10	92	0	1	0	2
Microfilaria rate (Mf)						0.89%	
Infection rate (PCR)						1.96%	

Note: A. Buaran. B. Doro. C. Kesesi. D. Wonokerto. E. Rirto. F. Kajen. G. Bojong. H. Siwalan. I. Kedungwuni. I. Wiradesa.

Table 2. Number of positive pools of surgery and PCR assay of Cx. quinquefasciatus, Ae. aegypty and Ae. albopictus collected in 10 location of Pekalongan Regency, Central Java, Indonesia

No	Location Code	*Cx. Quinquefasciatus*			*Ae. Aegypti*			*Ae. albopictus*		
		Number of pools	Micros-copic	PCR	Number of pools	Micros-copic	PCR	Number of pools	Micros-copic	PCR
1	A	21	0	0	4	0	0	0	0	0
2	B	2	0	0	4	0	1	1	0	0
3	C	6	0	0	3	0	0	1	0	0
4	D	8	0	0	0	0	0	0	0	0
5	E	15	0	0	0	0	0	0	0	0
6	F	8	0	0	0	0	0	0	0	0
7	G	13	0	0	0	0	0	0	0	0
8	H	14	0	0	0	0	0	0	0	0
9	I	12	0	0	0	0	0	0	0	0
10	J	15	0	1	0	0	0	0	0	0
Σ		114	0	1	11	0	1	2	0	0
Infection rate (PCR)			0%	0.89%		0%	9.10%		0%	0%

Note: A. Buaran. B. Doro. C. Kesesi. D. Wonokerto. E. Rirto. F. Kajen. G. Bojong. H. Siwalan. I. Kedungwuni. J. Wiradesa.

5729 mosquitoes were collected (5152 females and 577 males). Identification based on the guidance12 obtained 8 species, namely *Culex quinquefasciatus*, *Culex vishnui*, *Culex tritaeniorhynchus*, *Aedes aegypti*, *Aedes albopictus*, *Anopheles subpictus*, *Anopheles vagus*, and *Armigeres kesseli*. Surgery was performed on as many as pools of female mosquitoes, and it showed negative results. PCR examination results on the pools of female mosquitoes of *Cx. quinquefasciatus*, *Ae. aegypti* and *Ae. albopictus* from each location obtained 1 positive pool of 11 pools of the *Ae. aegypti* (infection rate of 9.10%). The figure of electrophoresis is presented in Figure 1 and 2.

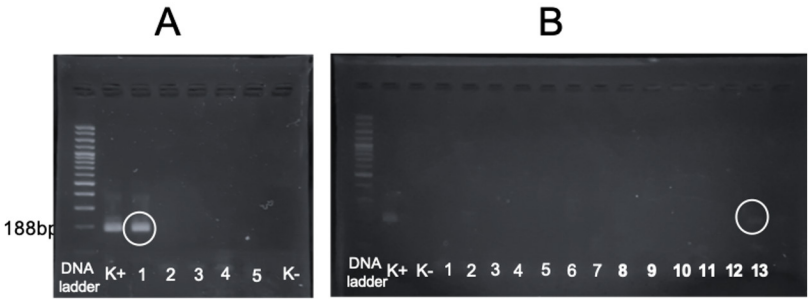

Figure 1. PCR amplification results of blood samples from 4 locations in Pekalongan Regency, Central Java, Indonesia. A.1.Buaran, B.13. Bojong

Figure 2. PCR amplification of the *Ae. aegypti* pool from Doro Pekalongan Regency, Central Java, Indonesia.

There was no larva of W. bancrofti on the microscopic dissecting mosquito of artificial infection at both day 8th and 14th. Likewise, the PCR examination results were negative at both stages of the examination.

DISCUSSION

Pekalongan is a district in the province of Central Java considered an filariasis endemic area. Amass preventive drug administration program had been implemented since 2015 for 5 years until 2019.[6,13] Its evaluation should have been conducted in 2020, but because of the Covid19 pandemic 2019, the evaluation cannot be conducted. So, the evaluation is planned to be conducted in 2021. This study conducted at 2019 found mf rate of 0.89%. This number is actually smaller than the endemicity rate (1%) set by WHO.[14] However, filariasis sufferers with microfilariae in their blood are a source of infection that can transmit filarial worms to other residents. The *W. bancrofti* microfilariae in blood samples was classified as high density, which was 416.67/mL. This finding indicated a density

of 2 times of the lowest standard in filariasis transmission, i.e. 200 mf/mL.[15] According to Hamilton and deMeilon cit Korte et al, (2013), they argued that transmission is not easy as it takes about 15.500 infective bites to produce microfilaremia.[16] In the communities of the location of the collection showed that there were abundant mosquitos and dominant *Cx. quinquefasciatus*. This mosquito was believed as a main vector for *W. bancrofti* in the urban areas.[4,17,18] It can be explain that in these areas have potentially of filariasis transmission due to the positive results in microscopic examination and the mf density in the non-elephantiasis patient, It could be noted that these areas have potentialities of filariasis transmission due to the positive results of microscopic examination and the mf density in the non-elephantiasis patients. It is possible that there are quite high sufferers and vectors, although the findings in this study were low.

Molecularly, it was found that the infection rate was 1.96%.The infection rate of 1.96% contained *W. bancrofti* without distinguishing its stages, whether microfilariae or adult worms. This did not indicate infectivity or endemicity of an area, but it indicated an individuals' potentiality as a source of infection by a presence of *W. bancrofti* DNA in their body. Therefore, this examination needs to be followed by sequence to determine appropriate and suitable species. This molecular finding might provide a picture that is in accordance with the Hamilton's theory that in fact there were microfilaremia sufferers at the study locatiosn, but they were not recruited as respondents. This was also supported by the finding of high *Cx. quinquefasciatus* mosquitoes that were caught. The high density of *Cx. quiquefasciatus* is in line with the results of previous studies.[19,20]

Therefore, it can be concluded that the *W. bancrofti* DNAs were found in *Cx. quinquefasciatus* and *Ae. aegypti* regardless of their stage whether microfilariae, larvae L1, L2 or L3. This means that *Ae. Aegepti* have a potentiality to become vectors of filariasis. It is known that urban type of bancroftian filariasis is transmitted by *Cx. quinquefasciatus* as a vector.[8,17] Until now roles *Ae. aegypti* have not yet confirmed as a vector of filariasis in Central Java; however, a Ramadhani's study on periodicity of *W. bancrofti* microfilariae found that 14% of microfilariae were still circulating in the peripheral blood at 6 am.[4] On the other hand, in the morning the Aedes mosquito has started to increase in density. These two phenomena allow transmission of filariasis by *Ae. aegypti*, where microfilariae are found in the blood and mosquito communities are found in the nature. In this study the PCR results on *Ae. aegypti* mosquitoes were 9.10%. With these findings, it is possible that *W. bancrofti* microfilariae can live in *Ae. aegypti* and develop into the infective L3 larval stage. It is known that *Ae. aegypti* is the main vector of dengue virus. So that this can be an input for the government for programs to eradicate animal-borne diseases, especially vector borne diseases. The eradication program can go hand in hand between the two diseases which can result in savings in funding.

Artificial infection against *Cx quinquefasciatus* and *Ae. aegypti* did not get positive results. However, this negative result does not simply invalidate the hypothesis about the potential of *Ae. aegypti* as a vector for filariasis. The limitation of this artificial test was that the infected blood was blood that had stayed 2 days, so that the viability of microfilariae was not optimal. Another limitation was that of the *Ae. aegypti* infected is a laboratory collection mosquito that had been reproduced many times in the laboratory. The suggestion for further research is that the infection is carried out on the same day, in conditions of good microfilaria viability, and the infected mosquitoes are mosquitoes from the research field. Other than that, this research needs to be continued with efforts to find *W. bancrofti* larvae to ensure their role as vector filariasis in Pekalongan, Central Java. It is necessary to do further research with sequencing to determine the right worm species

and dissecting of *Ae. aegypti* to determine its role as a vector of filariasis in Pekalongan.

CONCLUSION

This study revealed that Pekalongan regency showed low filariasis endemicity with mf rate of 0.89% and PCR infection rate of 1.96%. The microscopic examination of the mosquitos showed negative larvae although by PCR technique there were infection rates of 9.10% in *Ae. Aegypti*. Artificial infection of microfilaria of *W. bancrofti* against *Cx. quinquefasciatus* and *Ae. aegypti* was negative.

CONFLICT OF INTEREST

This study did not have any conflict of interest.

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