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 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 determine that Aedes 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 Aedes 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, Anopheles albopictus, Anopheles subpictus, Anopheles vagus, and Armigeres kesseli. Microfilarial (mf) rate was 0.89%, 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 Aedes aegypti 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.
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 One 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. 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. The Blood was collected at night starting at 8pm in one elephantiasis patient and 9 non-elephantiasis people in the vicinity. 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.

The mf rate (%) was calculated by formula:
\[
\text{mf rate} = \frac{\text{number of positive samples}}{\text{number of all samples}} \times 100
\]
while the microfilaria density (permL) was calculated by formula:
\[
\text{microfilaria density} = \frac{\text{number of microfilaria found in slide}}{\text{number of slide}} \times 16.67
\]

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 IF 5’-CCCTCTT ACC ATG AGC GAC-3’, and Ssp IR 5’-CCCTCA CTG ATG AGC GAC-3’). The PCR assay was performed by setting as follow:

| Step                     | Temperature | Time  |
|--------------------------|-------------|-------|
| Pre denaturation         | 94°C        | 5 minutes |
| Denaturing               | 94°C        | 1 minute |
| 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.

Data is presented in descriptive form.

Mosquitoes 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.

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:
\[
\text{larval density} = \frac{\text{number of larva found in pool}}{\text{number of pool}} \times 16.67
\]

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 elephantias | Elephantiasis | Non elephantias |
| 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. J. Wiradesa.

Table 2. Number of positive pools of surgery and PCR assay of *Cx. quinquefasciatus*, *Ae. aegypti* 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 | Microscopic | PCR | Number of pools | Microscopic | PCR | Number of pools | Microscopic | 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.
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. 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. 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. 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. 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. 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 microfilaria sufferers at the study location, but they were not recruited as respondents. This was also supported by the finding of high Cx. quinquefasciatus mosquitos that were caught. The high density of Cx. quinquefasciatus is in line with the results of previous studies.

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. Aegypti 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. 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. 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 mosquitoes 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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